บทบาทของแองจิโอเทนซินต่อการแสดงออกของยีนและโปรตีนไดไฮโดรโฟเลต รีดักเทส จีทีพีไซโคลไฮโดรเลส 1 และไนตริกออกไซด์ซินเธสในไต ในภาวะที่ไตขาดเลือดไปเลี้ยงชั่วคราว

นางสาวอยู่เย็น ซื้อจ้าง

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
สาขาวิชาสรีรวิทยา (สหสาขาวิชา)
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ปีการศึกษา 2549

ROLE OF ANGIOTENSIN IN RENAL DIHYDROFOLATE REDUCTASE, GTP-CYCLOHYDROLASE 1 AND NITRIC OXIDE SYNTHASE EXPRESSION IN RENAL ISCHEMIC REPERFUSION

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A Dissertation Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy Program in Physiology
(Interdisciplinary Program)
Graduate School
Chulalongkorn University
Academic Year 2006

Thesis Title	ROLE OF ANGIOTENSIN IN RENAL DIHYDROFOLATE REDUCTASE, GTP-CYCLOHYDROLSE 1 AND NITRIC OXIDE SYNTHASE EXPRESSION IN RENAL ISCHEMIC REPERFUSION
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อยู่เย็น ชื้อจ้าง: บทบาทของแองจิโอเทนซิน ต่อการแสดงออกของยีนและโปรตีนไดไฮโดรโฟเลตรีดักเทส จีทีพีไซโคลไฮโดรเลส 1 และไนตริกออกไซด์ซินเธสในไต ในภาวะที่ไตขาดเลือดไปเลี้ยงชั่วคราว (Role of angiotensin in renal dihydrofolate reductase, GTP-cyclohydrolase 1 and nitric oxide synthase expression in renal ischemic reperfusion) อ. ที่ปรึกษา : รศ. ดร. สมจิตร์ เอี่ยมอ่อง, อ. ที่ปรึกษาร่วม : อ. ดร. ทวีศักดิ์ ดีระวัฒนพงษ์, 91 หน้า.

ภาวะที่ไตขาดเลือดไปเลี้ยงชั่วคราว**เพิ่มระดับแองจิโอเทนซิน 2** (ANG II) การศึกษาจากการเพาะเลี้ยงเซลล์พบว่า แองจิโอเทนซิน 2 ลดระดับไดไฮโดรโฟเลตรีดักเทส (DHFR) **แต่ไม่เปลี่ยนแปลงระดับ**จีทีพีไซโคลไฮโดรเลส 1 (GTP-CH 1) พบว่าเอนไซม์ทั้งสองตัวนี้ถูก**ใช้เพื่อสร้าง**บีเอช 4 (BH4) **ซึ่งมี**บทบาทสำคัญต่อการเป็นโครงสร้างคู่ (dimer) ของไนตริกออกไซด์ซินเธส (NOS) แต่ยังไม่มี**การศึกษาบทบาทของ แองจิโอเทนซิน** 2 ต่อการแสดงออกพร้อมๆ กันของยีนและโปรตีนของไดไฮโดรโฟเลตรีดักเทส **จีทีพีไซโคลไฮโดรเลส 1 และไนตริกออกไซด์ซินเธส** ในภาวะที่ไตขาดเลือดไปเลี้ยงชั่วคราว การวิจัยครั้งนี้ทำการทดลอง**ในหนูแรทพันธุ์วิสต้าซึ่งแบ่งออก**เป็น 2 กลุ่มใหญ่ๆ คือ กลุ่ม sham (S) และกลุ่มที่**ชักนำให้เกิด**ภาวะขาดเลือดไปเลี้ยงชั่วคราว**ที่ไตข้างซ้าย โดยการอุดกั้น**หลอดเลือดเลี้ยงไตเป็นเวลา 30 นาที และให้เลือดไปเลี้ยงไตดังเดิมเป็นเวลา 1 วัน (IR) **ซึ่งทั้ง 2 กลุ่มนี้จะถูกแบ่ง**เป็นอีก 3 กลุ่มย่อย คือ 1) ได้รับน้ำดื่มเพียงอย่างเดียว หรือ 2) ได้รับน้ำดื่มผสม angiotensin converting enzyme inhibitor (ACEI; Enalapril®; 5 mg/kg/day) หรือ 3) ได้รับน้ำดื่มผสม angiotensin II receptor type 1 blocker (ARB; Losartan®; 10 mg/kg/day) สัตว์ทดลองจะได้รับสารดังกล่าว 1 **วันก่อนการผ่าตัดทำ** S **หรือ** IR **และรับต่อไปอีก**เป็นเวลา 1 **วันหลัง**การผ่าตัด เมื่อครบกำหนดการทดลอง ทำการเก็บ**ตัวอย่างปัสสาวะและเลือดเพื่อตรวจวัดระดับ**อิเลคโตรไลต์ **สารยูเรียไนโตรเจน** ครีเอตินีน และคำนวณค่า**ครีเอตินีนเคลียแรนซ์ รวมทั้งเก็บตัวอย่างเนื้อไต เพื่อแยก** RNA **และตรวจวัดระดับ**การแสดงออกของยีน (mRNA) และโปรตีนของไดไฮโดรโฟเลตรีดักเทส **จีทีพีไซโคลไฮโดรเลส 1 เอนโดธีเลียลและอินดิวซิเบิลไนตริกออกไซด์ซินเธส** โดยวิธี Reverse transcriptase polymerase chain reaction (RT-PCR) **และ** Western blot ตามลำดับ ผลการทดลองพบว่า IR ลดระดับ**ไดไฮโดรโฟเลตรีดักเทส ทั้งการแสดงออกของยีนและโปรตีน** ($p<0.01$) ซึ่งมีค่าเพิ่มขึ้นเมื่อให้ ACEI ($p<0.05$) หรือ ARB ($p<0.01$) **ในขณะที่ระดับจีทีพีไซโคลไฮโดรเลส 1 มีค่าเท่าเดิม** IR ลดระดับเอนโดธีเลียลไนตริกออกไซด์ซินเธส**โครงสร้างคู่ ($p<0.01$) แต่เพิ่มระดับเอนโดธีเลียลไนตริกออกไซด์ซินเธสโครงสร้างเดี่ยว** (monomer) ($p<0.01$) ซึ่งระดับดังกล่าว**มีค่ากลับคืนเมื่อให้ ACEI หรือ ARB ($p<0.01$) ระดับการแสดงออกของยีนและโปรตีนรวมของเอนโดธีเลียลไนตริกออกไซด์ซินเธส** (total eNOS protein) **มีค่าไม่เปลี่ยนแปลงในทุกกลุ่มการทดลอง แต่ IR เพิ่มระดับ**การแสดงออกของยีน โปรตีนรวม **และโครงสร้างเดี่ยวของอินดิวซิเบิลไนตริกออกไซด์ซินเธส** ($p<0.01$) **ซึ่งมีระดับลดลงโดย** ACEI หรือ ARB ($p<0.01$)

การศึกษาครั้งนี้แสดงข้อมูลชิ้นแรกที่บ่งว่า ภาวะที่ไตขาดเลือดไปเลี้ยงชั่วคราว โดยมีการกระตุ้นผ่านแองจิโอเทนซิน 2 นั้น ลดระดับไดไฮโดรโฟเลตรีดักเทส แต่เพิ่มอินดิวซิเบิลไนตริกออกไซด์ซินเธส ทั้งการแสดงออกของยีนและโปรตีน ภาวะ IR เพิ่มระดับโครงสร้างเดี่ยว แต่ลดระดับโครงสร้างคู่ของเอนโดธีเลียลไนตริกออกไซด์ซินเธส การยับยั้งแองจิโอเทนซิน 2 ด้วย ACEI หรือ ARB สามารถเพิ่มระดับไดไฮโดรโฟเลตรีดักเทสและเอนโดธีเลียลไน ตริกออกไซด์ซินเธสโครงสร้างคู่ ซึ่งในขณะเดียวกันสามารถลดระดับอินดิวซิเบิลไนตริกออกไซด์ซินเธสและไนตริกออกไซด์ซินเธสโครงสร้างเดี่ยวที่เพิ่มสูงขึ้น พบว่า IR ไม่มีผลต่อจีทีพีไซโคลไฮโดรเลส 1 การศึกษานี้ยังบ่งชี้อีกด้วยว่า ANG II receptor type 1 แสดงบทบาทสำคัญในการควบคุมการแสดงออกของ ไดไฮโดรโฟเลตรีดักเทส และไนตริกออกไซด์ซินเธส

ภาควิชา	สรีรวิทยา	ลายมือชื่อนิสิต.....
สาขาวิชา	สหสาขาวิชาสรีรวิทยา	ลายมือชื่ออาจารย์ที่ปรึกษา.....
ปีการศึกษา	2549	ลายมือชื่ออาจารย์ที่ปรึกษาร่วม .....

4689695620: MAJOR PHYSIOLOGY
KEY WORD: ANGIOTENSIN/ DIHYDROFOLATE REDUCTASE/ GTP-CYCLOHYDROLASE 1/ NITRIC OXIDE SYNTHASE/ RENAL ISCHEMIC REPERFUSION

YUYEN SEUJANGE: ROLE OF ANGIOTENSIN IN RENAL DIHYDROFOLATE REDUCTASE, GTP-CYCLOHYDROLASE 1 AND NITRIC OXIDE SYNTHASE EXPRESSION IN RENAL ISCHEMIC REPERFUSION. THESIS ADVISOR: ASSOC. PROF. SOMCHIT EIAM-ONG, Ph.D., THESIS CO-ADVISOR: THAWEESAK TIRAWATNAPONG, Ph.D., 91 pp.

Renal ischemic reperfusion (IR) enhances angiotensin II (ANG II) level. In cell culture study, ANG II suppresses dihydrofolate reductase (DHFR) but not modulate GTP-cyclohydrolase I (GTP-CH 1) expression. Both enzymes are required for synthesis of BH4 which plays a crucial role in dimerization of nitric oxide synthase (NOS). There is no simultaneous study the role of ANG II on renal DHFR, GTP-CH 1, NOS gene and protein expression during IR. Male Wistar rats were divided into two main groups: sham (S) or IR (30-minute renal pedical occlusion and reperfusion for 1 day). Both groups were further treated with 1) water, or 2) angiotensin converting enzyme inhibitor (ACEI; Enalapril®; 5 mg/kg/day), or 3) angiotensin II receptor type 1 blocker (ARB; Losartan®; 10 mg/kg/day) for one day before S or IR and continuously for 1 day after the operation. On each experimental due date, 24-hr urine and blood samples were collected. The serum was determined for electrolytes, blood urea nitrogen, creatinine (Cr), and Cr clearance (CCr). The kidneys were removed for RNA isolation, detection of mRNA expression (RT-PCR), and protein abundance (Western blot) for DHFR, GTP-CH 1, endothelial NOS (eNOS), and inducible NOS (iNOS). The data show that IR decreased DHFR mRNA and protein levels ($p<0.01$) which were restored by ACEI ($p<0.05$) or ARB ($p<0.01$); whereas GTP-CH 1 expression was remained. IR suppressed the eNOS dimer ($p<0.01$) while enhanced the monomer ($p<0.01$) which were corrected by ACEI or ARB ($p<0.01$). The renal eNOS mRNA and total eNOS protein levels did not change in all experimental groups. IR increased iNOS mRNA, total iNOS protein, and iNOS monomer ($p<0.01$) which were reduced by ACEI or ARB ($p<0.01$).

The present study demonstrates the first evidence indicating that IR, via stimulation of ANG II, suppresses renal DHFR but activates iNOS both mRNA and protein expression. IR enhances the monomer while diminishes the dimer form of eNOS. Inhibition of ANG II by using ACEI or ARB could restore DHFR and eNOS dimer whereas attenuate the heightened levels of iNOS expression and NOS monomer. IR has no effect on GTP-CH 1 expression. The present result also indicates that angiotensin II receptor type 1 plays a crucial role in modulation of DHFR and NOS expression.

Field of study Physiology
Academic year 2006

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ACKNOWLEDGEMENT

This dissertation was carried out successfully through many valuable advice, helpful guidance, suggestion and intensive review from my advisor, Associate Professor Somchit Eiam-Ong, Ph.D., Department of Physiology and my co-advisor, Thaweesak Tirawatnapong, Ph.D., Department of Microbiology, whom I would like to express my deep gratitude.

My sincere and appreciation is also expressed to Professor Somchai Eiam-Ong, M.D., and Assistant Professor Sarinee Kalandakanond-Tongsong, D.V.M., Ph.D. for their suggestions of some techniques in preliminary work. Grateful acknowledgement is also expressed to Department of Physiology, Department of Microbiology, Division of Nephrology, Department of Medicine, and Chulalongkornn Medical Research Center (Chula MRC), Faculty of Medicine, Chulalongkorn University for provision the facilities used in experimental works. I would like to express my sincere thanks to the Chairman, Associate Professor Prasong Siriviriyakul, M.D., and my thesis committee, Professor Prasit Futrakul, M.D., and Associate Professor Chollada Buranakarl, D.V.M., Ph.D. for their valuable comments, suggestions and corrections of this dissertation.

I am also indebted to all experimental rats for their sacrifice which bring me to succeed in my study. Finally, I am extremely grateful to my family for their love, understanding and encouragement throughout my graduate study. I would like to special thank to Miss Niramol Thammacharoenrach, Miss Rapeeporn Mannontarat, Miss Sirigul Kanchanabuch, Miss Worakamol Naen-Udorn, and all of my friends for their helps and cheerfulness.

There is no doubt that my study could not be completed without the support from The Ratchadapiseksompoth Research Fund, Faculty of Medicine, and the scholarship from The Graduate School, Chulalongkorn University.

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LIST OF ABBREVIATIONS

ACE	Angiotensin converting enzyme
ACEI	Angiotensin converting enzyme inhibitor
ANG II	Angiotensin II
ARB	Angiotensin II receptor type 1 blocker
ARF	Acute renal failure
AT1	Angiotensin II receptor type 1
AT2	Angiotensin II receptor type 2
BH4	5,6,7,8-Tetrahydrobiopterin
BH2	7,8-dihydrobiopterin
BUN	Blood urea nitrogen
Cr	Creatinine
CCr	Creatinine clearance
cDNA	Complementary deoxyribonucleic acid
°C	Degree Celsius
DHFR	Dihydrofolate reductase
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphates
eNOS	Endothelial nitric oxide synthase
GAPDH	Glyceroldehyde-3-phosphate-dehydrogenase
GFR	Glomerular filtration rate
FAD	Flavin adenine dinucleotide
FMN	Flavin mononucleotide
GTP-CH 1	GTP-Cyclohydrolase 1
H_2O_2	Hydrogen peroxide

iNOS	Inducible nitric oxide synthase
IFN-γ	Interferon-γ
IR	Ischemic reperfusion
LPS	Lipopolysaccharide
LT-PAGE	Low temperature sodium dodecyl sulfate-polyacrylamide gel electrophroresis
MAP	Mean arterial pressure
M	Molar
M-MLV	Malony murine leukemia virus
mRNA	Messenger ribonucleic acid
NADPH	nicotinamide adenine dinucleotide phosphate (oxidized form)
NO	Nitric oxide
NOS	Nitric oxide synthase
nNOS	neuronal nitric oxide synthase
$ONOO^{\bullet}$	Peroxynitrite
$O_2^{\bullet}$	Superoxide
RBF	Renal blood flow
RT-PCR	Reverse transcriptase polymerase chain reaction
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophroresis
VR	Vascular resistance
μl	Microlitre

CHAPTER I

INTRODUCTION

BACKGROUND AND RATIONALE

Renal ischemic reperfusion (IR) is characterized by a decline in renal blood flow (RBF) and oxygen supply (Waz et al., 1998). This often occurs during renal transplantation, surgical revascularization of the renal artery, resuscitation from hypotension states, and treatment of suprarenal aortic aneurysms (Rhoden et al., 2001).

In renal IR, a reduction in blood pressure causes renin release (Allred et al., 2000; Bivol et al., 2005), and also increases angiotensin converting enzyme (ACE) activity (Guan et al., 1992), then enhances angiotensin II (ANG II) levels (Allred et al., 2000; Kontogiannis and Burns, 1998). In addition to its hemodynamic effects directly, ANG II could affect nitric oxide (NO) production, NO synthase (NOS) expression and function via alteration of tetrahydrobiopterin (BH4) synthesis (Chalupsky and Cai, 2005; Hennington et al., 1998). BH4 is one of essential co-factors for dimerization of NOS to be an active form (Kakoki et al., 2000). Deficiency of BH4 causes NOS uncoupling which produces superoxide ($O_2^{\cdot}$) instead of NO (Chalupsky and Cai, 2005; Kakoki et al., 2000).

Generally, BH4 could be produced by de novo or salvage pathway (Thony et al., 2000). The former uses GTP-cyclohydrolase 1 (GTP-CH 1) whereas the latter requires dihydrofolate reductase (DHFR) (Thony et al., 2000). Recently, Chalupsky and Cai (2005) have demonstrated that ANG II rapidly and transiently activates NADPH oxidase to produce $O_2^{\cdot}$, leading to hydrogen peroxide (H_2O_2)-dependent

down-regulation of DHFR in bovine aortic endothelial cell culture (BAEC). In addition, a second phase of $O_2^{\cdot}$ production from uncoupled NOS has also been noted (Chalupsky and Cai 2005). The overexpression of DHFR could restore NO production and abolish $O_2^{\cdot}$ production from uncoupled NOS in ANG II stimulated cells (Chalupsky and Cai, 2005). These data characterize an essential role of DHFR in maintaining BH4 and NO bioavailability, and ANG II also modulates DHFR under this circumstance. However, the exogenous H_2O_2 could up-regulate GTP-CH 1 expression which increases total biopterin, but the endogenous H_2O_2 produced by ANG II does not modulate GTP-CH 1 (Chalupsky and Cai, 2005; Shimizu et al., 2003). These results indicate that DHFR is much more sensitive to lower endogenous H_2O_2 compared to GTP-CH 1, which is regulated only in response to exogenous H_2O_2.

As noted above, during renal IR a rise in ANG II occurs. This would modulate the expression of either DHFR or GTP-CH 1, or both and subsequently influence on NOS expression. However, there is no study on IR in the relation to renal DHFR, GTP-CH 1, and NOS expression simultaneously as well as the role of ANG II and ANG II receptor type 1 in this circumstance. Therefore, the present study aims to investigate this regard.

RESEARCH QUESTIONS

1. Does IR have any effects on renal DHFR, GTP-CH 1, eNOS and iNOS expression?
2. Does angiotensin inhibition has an influence on renal DHFR, GTP-CH 1, eNOS and iNOS expression in IR? If so, is it mediated via angiotensin receptor type 1?

RESEARCH OBJECTIVES

1. To study the changes of renal DHFR, GTP-CH 1, eNOS and iNOS expression in IR.
2. To study the role of angiotensin in renal DHFR, GTP-CH 1, eNOS and iNOS expression in IR.
3. To study the role of angiotensin receptor type 1 in the changes of renal DHFR, GTP-CH 1, eNOS and iNOS expression in IR.

HYPOTHESIS

1. IR would decrease renal DHFR and eNOS expression but increase GTP-CH 1 and iNOS.
2. Angiotensin inhibition could restore those changes of renal DHFR, GTP-CH 1, eNOS and iNOS expression in IR.
3. Angiotensin receptor type 1 involves in the changes of renal DHFR, GTP-CH 1 eNOS and iNOS expression in IR.

KEY WORDS

angiotensin

dihydrofolate reductase

GTP-cyclohydrolase 1

nitric oxide synthase

renal ischemic reperfusion

EXPECTED BENEFIT AND APPLICATION

The results would provide further supporting data for the role of angiotensin on DHFR, GTP-CH 1, eNOS, and iNOS expression in renal IR model.

CHAPTER II

THEORY AND LITERATURE REVIEW

RENAL ISCHEMIC REPERFUSION

Renal ischemic reperfusion (IR) occurs in clinical practice and is a consequence of systemic hypoperfusion with subsequent circulatory resuscitation (Jefayri et al., 2000). Local renal hypoperfusion after aortic cross clamping or renal transplantation also causes IR injury to the kidney (Jefayri et al., 2000). Renal IR is an important cause of renal dysfunction often leading to acute renal failure (ARF), causing high mortality among patients in intensive care who require dialysis (Deng et al., 2001). The severity of the injury depends on the duration of ischemia and subsequent reperfusion (Aragno et al., 2003). Reperfusion, although essential for the survival of ischemic renal tissue, causes additional damage (Aragno et al., 2003).

A decrease in RBF is of critical importance in initiating and extending the pathophysiology of ischemic ARF (Molitoris and Sutton, 2004). Under physiologic conditions, the oxygen tension of the kidney decreases as one moves from the outer cortex to the inner medulla (Brezis and Rosen, 1995). Regional alterations in RBF persist after the ischemic event and play an important role in the extension phase or renal ischemic injury. During reperfusion a reduction in total RBF of 40% to 50% of normal has been reported in both animal models of ischemic ARF and in human ischemic ARF (Lieberthal, 1997). Studies have demonstrated a persistent reduction in RBF contributes significantly to the diminished glomerular filtration rate (GFR) observed in human allografts following ischemic ARF (Alejandro et al., 1995). Mechanisms involved in the alteration of renal perfusion following ischemic injury

are incompletely understood. An imbalance between mediators of renal vasoconstriction and renal vasodilatation has been proposed to play a role in animal models of ischemic ARF. The two molecules that have been widely studied and very implicated in renal IR injury are ANG II and NO.

ANGIOTENSIN II

ANG II is a potent vasoconstrictor that produced by renin angiotensin cascade (Levens et al., 1992). The enzyme renin is secreted by the kidney into the blood in response to a number of stimuli, including a reduction in blood pressure and a reduction in extracellular fluid volume. Renin cleaves 10 amino acids from its α-globulin substrate angiotensinogen to form the decapeptide ANG I. ANG I is rapidly converted to the biologically active octapeptide (ANG II) upon circulation through organs, by the action of the endothelial enzyme ANG converting enzyme (ACE) (Levens et al., 1992).

Generation of ANG II acts to constrict vascular smooth muscle cells and also modulates aldosterone secretion, cathecholamine release and secretion of pituitary peptides and hormones (Wong et al., 1990). Within the kidney, ANG II modulates GFR by exerting direct effects on afferent and efferent arteriolar tone and on mesangial cell function (Ichikawi and Harris, 1991). The proximal tubule (PT) contains mRNA for all components of the renin angiotensin system. High levels of ANG II are present in proximal tubular lumen, this suggests that ANG II is synthesized locally and can act in an autocrine or paracrine fashion on tubular cells (Kontogiannis and Burns, 1998). ANG II mediates its effect in the renal vasculature through two main G-coupled receptors, the AT1 and AT2 receptors (Bivol et al., 2005; Murphy et al., 1991). The rat has two types of AT1 receptors, the $AT1_A$ and

$AT1_B$ receptors, which have 95% homology in their receptor amino acid sequences (Kakar et al., 1992). However, in the human kidney, only a single gene encoding for the AT1 receptor is expressed (Curnow et al., 1992). AT1 receptor distribution is widespread in the kidney such as in the vasculature on both smooth muscle plasma membranes and endothelial cells including afferent and efferent arterioles (Ardaillou, 1999) as well as in the PT (Gunning et al., 1996). The AT2 receptor has only 34% homology with the AT1 receptor and is expressed at the low level, particularly in the renal vasculature (Douglas and Hopfer, 1997). Moreover, the localization and function of AT2 receptor in the adult kidney remain unclear (Kontogiannis and Burns, 1998).

In IR condition, a reduction in blood pressure causes renin release (Allred et al., 2000; Bivol et al., 2005), and also increases ACE activity (Gaun et al., 1992), subsequently enhances ANG II levels (Allred et al., 2000; Kontogiannis et al., 1998). The role of ANG II during IR is extraordinary complex. Generation of ANG II acts to raise perfusion pressure and to protect glomerular filtration by efferent arteriolar constriction (Lerman and Textor, 2001). Because the efferent arteriole constricts at lower ANG II concentrations (10^{-11} M) than the afferent arteriole (10^{-9} M) (Ito et al., 1993). This will primarily constrict the efferent arteriole but possibly also the afferent arteriole if ANG II increases sufficiently (Sorensen et al., 2004). The rat model with IR shows renal damage, increased vascular resistance (VR) and mean arterial pressure (MAP) as well as decreased RBF (Sadjadi et al., 2002; Vargas et al., 1994). ACE inhibitor (ACEI) (Vargas et al., 1994) or AT1 receptor blocker (ARB) (Barrilli et al., 2004; Kontogiannis and Burns, 1998) could ameliorate renal damage, decrease VR and MAP and also increase RBF (Bivol et al., 2005; Vargas et al., 1994). The results suggest that ANG II acting via the AT1 receptor plays a role in the

development of renal damage after IR. In addition to its hemodynamic effects directly, the recent study have demonstrated that ANG II could affect NO production, NOS expression and function via alteration of BH4 synthesis (Chalupsky and Cai, 2005; Hennington et al., 1998).

NITRIC OXIDE AND NITRIC OXIDE SYNTHASES

NO is a free radical gas that could diverse biomessenger function and participate in the regulation of kidney function by counteracting the vasoconstrictor effects of ANG II (Lerman and Textor, 2001). NO is a potent activator of guanylate cyclase, an important hemo-protein target of NO (Lane and Gross., 1999). Binding of NO alters the conformation of the heme moiety and causes activation of the enzyme. The subsequent rise in cGMP levels causes the activation of cGMP-dependent kinases and also augment Ca^{2+}-extrusion through the plasma membrane Ca^{2+}-ATPase (Masden et al., 2000). These result in vascular smooth muscle cells relaxation, suppression of platelet adhesion and aggregation (Azuma et al., 1986; Masden et al., 2000).

NO is synthesized from L-arginine by a family of NOS (Schwobel et al., 2000). There are three distinct isoforms of the enzyme (Marsden et al., 2000), nNOS (Bredt and Snyder, 1990; Mayer et al., 1990), eNOS (Lamas et al., 1992; Pollock et al., 1991), which partake in signalling cascades, are constitutively expressed and depend on the presence of Ca^{2+} for activity (Lane and Gross, 1999). However, the recent study has demonstrated that cNOS activity could be activated via either Ca^{2+}-dependent or Ca^{2+}-independent pathway (Cai et al., 2003). For example, H_2O_2 causes an acute and potent NO release from endothelial cells that is mediated by additive effects of the phosphatidylinositol 3-kinase/Akt-dependent eNOS serine 1179

phosphorylation and MEK/ERK ½ activation (Cai et al., 2003). In the kidney, the expression of nNOS is limited mainly to the macula densa and its regulatory role in signal transduction within the juxtaglomerular apparatus (Mundel et al., 1992). The eNOS is constitutively expressed by the endothelial lining in blood vessels of different caliber (Roczniak et al., 2000; Kone, 1997). The iNOS isoform is involved in the nonspecific immune response, regulated transcriptionally, and only expressed in response to cytokines, once expressed, it remains active in the absence of Ca^{2+} and will sustain continuous NO synthesis for days (Hevel et al., 1991; Stuehr et al., 1991; Lane and Gross, 1999). Surprisingly, iNOS is also expressed constitutively in several segments of the renal tubule as well as in the glomerulus, interlubar and arcuate arteries of the normal rat kidney (Mattson et al., 2000).

ANGIOTENSIN II AND NITRIC OXIDE SYNTHASES

The effects of ANG II on regulation of NOS and NO were extensively performed both in vitro and in vivo studies. Incubation of ANG II in various cell cultures alters NO production, NOS activity, and NOS expression. For example, in cultured bovine pulmonary artery endothelial cell (BPAE), eNOS mRNA was significantly increased 2.4-fold 6 h after the additional of ANG II (10^{-6} M) over basal levels. In a similar time course, it was found that eNOS protein concentrations are increased 247% over basal levels at 4 h after ANG II addition. There is a second protein peak at 8 h after ANG II treatment in which eNOS was increased 333% over basal (Olson et al., 1997). These data suggest that ANG II stimulate eNOS mRNA expression and are followed by increased levels of eNOS protein in cultured BPAE cells, consistent with an observed increase in nitrite (NO metabolite) production (Olson et al., 1997). Both the increase in eNOS protein and mRNA expression could

be attenuated with the ARB (saralasin) (Olson et al., 1997). Moreover, Olson et al. (2004) further demonstrated that ANG II could activate eNOS protein expression as a dose- and time-dependent, as well as activate eNOS mRNA expression and NO production in cultured BPAE cells. However, PD-123319, an AT2 receptor blocker could attenuate the heightened levels (Olson et al., 2004). These data indicate that ANG II activated eNOS expression and NO generation via both AT1 and AT2 receptor. These simulatory effects have also been observed in coronary microvascular endothelial cell (Bayraktutan and Ulker, 2003) and in ovine fetoplacental artery endothelium (Zheng et al., 2005).

Regarding to in vivo studies, an infusion of ANG II increased eNOS protein expression in myocardium via AT1 receptor (Tambascia et al., 2001). On the contrary, the eNOS mRNA and protein levels as well as NOS activity in the left ventricular are markedly decreased in the rat that given ANG II (200 ng/kg/min) for two weeks. The levels are significantly ameliorated by ARB, TCV-116 (Kobayashi et al., 2001). In the kidney, more intricate data have been noted. ANG II administration in rat increases NO production, NOS activity, and NOS expression (Chin et al., 1999; Zou et al., 1998). However, these alterations are variable and seem to depend on the period of ANG II exposure. For example, an acute ANG II infusion (110 minutes) increases eNOS mRNA in kidney by 70% without changing eNOS protein levels, whereas a chronic infusion (10 days) enhances eNOS protein by 90% without changing eNOS mRNA levels (Hennington et al., 1998). Moreover, Moreno et al. (2002) have also shown that ANG II infusion for 3 h increases renal cNOS activity without modification of their protein expression while a chronic ANG II (3 d) could augment the protein expression but not the activity. Furthermore, action of ANG II in the kidney is likely to be area specific regulation on NOS expression. For instance,

ANG II administration could up-regulate eNOS mRNA and protein in renal cortex, but not in renal medulla (Chin et al., 1999; Navar et al., 2000).

Moreover, the recent study has also demonstrated the effect of ANG II on iNOS expression. For example, in the transgenic rats that over expressed both human renin and angiotensinogen genes (dTGR) feature ANG II-induced iNOS protein expression (Theuer et al. 2005). In addition, an infusion of ANG II enhances iNOS mRNA level in aortic artery by activating of NF-κB cascade (Tham et al., 2002). Blocking ANG II by using losartan, an AT1 receptor blocker, can have protective effects on the renal injury in glomerulosclerosis by down-regulating the expressions of iNOS (Ji et al., 2005). The data indicate that ANG II could modify eNOS and iNOS at either a transcriptional or translational level.

Besides the effects on NOS expression and NO production, ANG II could increase $O_2^{\cdot}$ generation through activating membrane associated NADH/NADPH oxidase activity (Berry et al., 2000; Ushio-Fukai et al., 1996). The excessive $O_2^{\cdot}$ can interact with NO, which reduces NO bioavailability to generate peroxynitrite ($ONOO^-$), a potent oxidative stress (Walker et al., 2000). Moreover, the negative charged $O_2^{\cdot}$ radical is unstable in aqueous solution (half-life of a few seconds) and is rapidly dismutated to H_2O_2 (Li et al., 2004). Interestingly, the recent study in BAEC has demonstrated that H_2O_2 produced by ANG II could affect NOS function via changing in BH4 synthesis (Chalupsky and Cai, 2005). BH4 is one of essential co-factors for dimerization of NOS to be an active form (Kakoki et al., 2000; Thony et al. 2000). Deficiency of BH4 causes NOS uncoupling which produces $O_2^{\cdot}$ instead of NO (Chalupsky et al., 2005; Kakoki et al., 2000).

TETRAHYDROBIOPTERIN AND NITRIC OXIDE SYNTHASES

BH4, an essential co-factor for various processes, and is present in probably every cell or tissue of higher organisms (Thony et al., 2000). BH4 is required for various enzyme activities, and for less defined functions at the cellular level (Thony et al., 2000). One of the most investigated functions of BH4 is its action as a co-factor of NOS (Thony et al., 2000).

As mentioned earlier, all three NOS isoforms are the enzymes that catalyzes the production of NO and L-citrulline from L-arginine, O_2, and NADPH-derived electrons (Channon, 2004; Mayer and Hemmens, 1997). NOS utilizes six different co-factors and prosthetic groups: thiolate-bound heme (Klatt et al., 1992; Stuehr and Ikeda-Saito, 1992), FAD and FMN (Hevel et al., 1991; Stuehr et al., 1991), calmodulin/Ca^{2+} (Cho et al., 1992; Mayer et al., 1989), BH4 (Mayer et al., 1990; Pollock et al., 1991), and Zn^{2+} (Hemmens et al., 2000; Miller et al., 1999) (Figure A)

The enzyme functions as a dimer consisting of two identical monomer (Figure B, left side), which can be functionally and structurally divided into two major domain, a C-terminal reductase domain and N-terminal oxygenase domain (Figure A and B) (Channon, 2004; Hemnens and Mayer, 1998). The former contains binding sites for one molecule each of NADPH, FAD, and FMN, whereas the latter binds heme and BH4, as well as the substrate L-arginine (Andrew and Mayer, 1999). Between these two regions lies the calmodulin (CaM) binding domain, which plays a key role in both the structure and function (Figure A) (Andrew and Mayer, 1999).

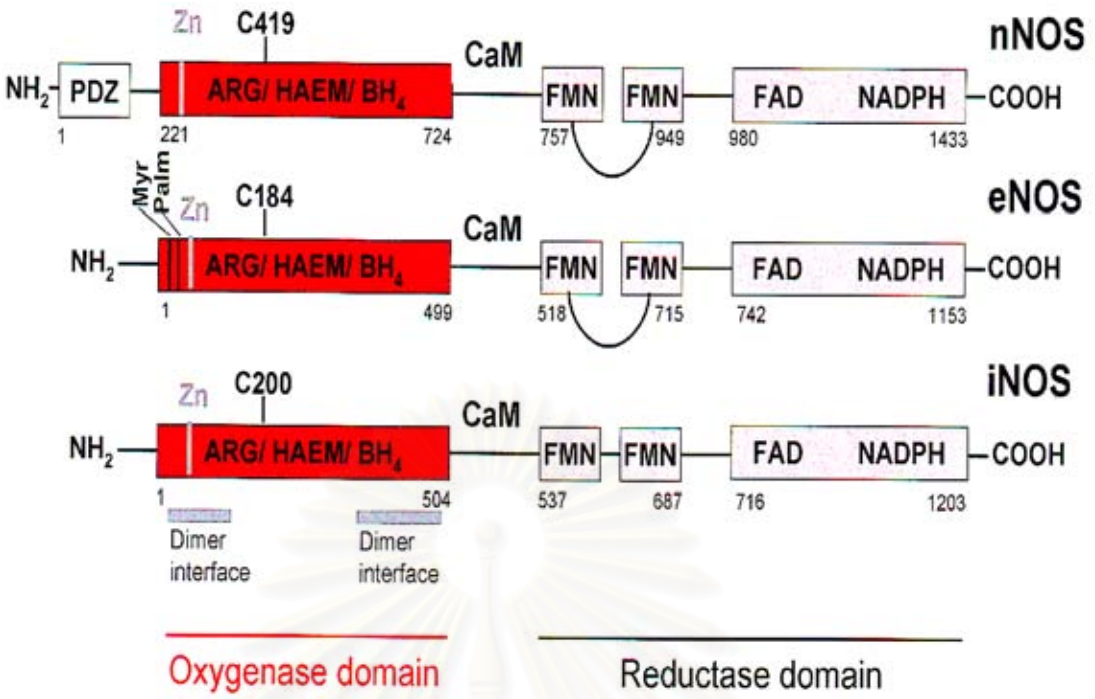


Figure A: The domain structure of human nNOS, eNOS and iNOS. Oxygenase, reductase and PDZ domains are denoted by solid boxes and the amino acid residue number at the start/end of each domain is shown. The cysteine residue which ligates the haem and the CaM-binding site is indicated for each isoform, myristoylation (Myr) and palmitoylation (Palm) sites on eNOS are shown, as is the location of the zinc-ligating cysteines (Zn in grey). The autoinhibitory loop within the FMN regions of nNOS and eNOS are also shown and grey bars indicate the dimer interface in the oxygenase domain.

NOS catalyzes the formation of NO in two consecutive steps, as shown in Figure B (right side) (Stuehr et al., 1991). First, L-arginine is oxidized to N^G-hydroxy-L-arginine (NHA), in a reaction that consumes one molecule of O_2 and two NADPH-derived electrons. Subsequently, NHA is converted into L-citrulline and NO with consumption of one more NADPH-derived electron and another molecule of O_2. This affords an overall stoichiometry of 1.5 NADPH oxidized per NO produced (Gorren et al., 2000; Griffith and Stuehr, 1995).

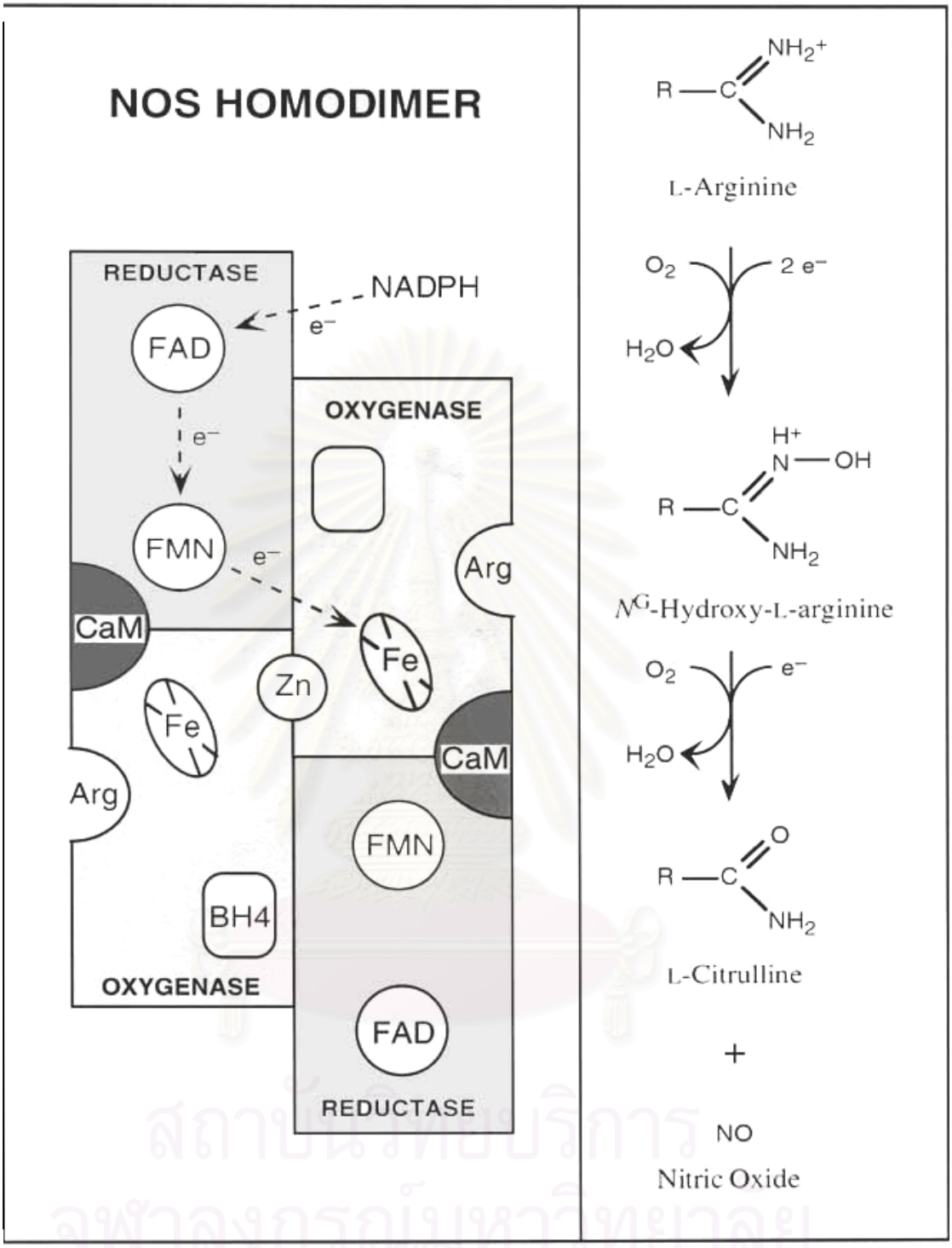


Figure B: Schematic structure of NOS. On the left side the homodimeric arrangement, domain structure, and distribution and stoichiometry of prosthetic groups and cofactors are illustrated. The electron transfer pathway from NADPH to one of the hemes is also indicated. On the right side the 2-step transformation of L-arginine into L-citrulline and NO is presented.

The catalytic center of NOS is a heme that is located in the oxygenase domain and heme has a cysteinyl thiolate for an axial ligand (Ghosh and Stuehr, 1995; Sheta et al., 1994). The substrate arginine binds close to the heme but without direct interaction (Fischmann et al., 1999; Raman et al., 1998). The function of the flavins is the same as in cytochrome P450 reductase. Electrons are transferred from NADPH via FAD and FMN to the heme in the oxygenase domain. FAD accepts two electrons at a time, but transfers them one by one to the FMN semiquinone, with reduced FMN functioning as a one electron donor to the heme (Noble et al., 1999). Calmodulin conveys a conformational change that is necessary for interdomain electron transfer (Abu-Soud and Stuehr, 1993; Sheta et al., 1994), and that also facilitates intradomain electron transfer, *i.e.* from FAD to FMN (Abu-Soud et al., 1994). This renders the constitutive isoforms sensitive to Ca^{2+} (Lamas et al., 1992; Mayer et al., 1990), in accordance with their role in cell signalling. The much higher affinity for calmodulin of iNOS enables this isoform to remain active in the absence of Ca^{2+} (Cho et al., 1992). The latest addition to the list of cofactors, Zn^{2+}, binds to NOS in a stoichiometry of 1 per dimer, where it is coordinated to four cysteinyl sulfur atoms (2 from each monomer) in the dimer interface (Fischmann et al., 1999; Li et al., 1999). It plays an important role in dimer stabilization, but is not essential for catalysis (Hemmens et al., 2000; Miller et al., 1999). BH4 binds to the oxygenase domain in the immediate vicinity of the heme and the substrate arginine, though not in the distal heme pocket. Instead it is bound near the dimer interface, and its binding site encompasses residues from both subunits (Crane et al., 1998; Raman et al., 1998). It has become clear that BH4 is essential for catalysis and its function is more and more elucidated.

All three NOS isoforms appear to be active only in its dimeric form (Alderton et al., 2001). Dimerization created between two oxygenase domains of NOS is one of several posttranslational steps required for conversion into an active enzyme (Siddhanta et al., 1996). NOS dimerization is initiated by heme insertion, which results in rapid conformational changes (Klatt et al., 1996). The heme-containing NOS monomer is an intermediate in dimerization and in the presence of BH4 and L-arginine, form a stable active dimer (Figure B) (Ghosh et al., 1996).

A critical aspect of NOS function is the requirement for the co-factor BH4 (Channon, 2004). BH4 binds close to the heme active site at the interface between the two monomers, interacting with residues from both monomers (Figure B) (Raman et al., 1998). Maintenance and stabilization of NOS dimers is dependent on BH4, which plays a direct role in the multiple step oxidation of arginine through the N^{G}-hydroxyl-L-arginine intermediate and the subsequent generation of NO (Channon, 2004).

When BH4 is limiting or absent, NOS biochemistry is fundamentally altered in a number of ways, not merely by a loss of enzymatic activity. First, NOS dimerization is destabilized, leading to a reduction in the relative proportion of NOS dimers versus monomers present in the cell. Second, NOS catalytic activity becomes uncoupled (Channon, 2004). In this situation, the stoichiometric coupling between the reductase domain and L-arginine oxidation at the active site is lost. However, electron transfer from NADPH through the flavins to molecular oxygen is not inhibited, but results in a formation of $O_2^{\bullet}$ and/or H_2O_2 instead of NO (Chalupsky and Cai, 2005; Channon, 2004). BH4 appears necessary, even not always sufficient to prevent NOS uncoupling.

TETRAHYDROBIOPTERIN SYNTHESIS

BH4 is synthesized de novo from guanosine triphosphate (GTP) by the enzymes GTP-CH 1, 6-pyrovoyl-tetrahydrobiopterin synthase (PTPS), and sepiaterin reductase (SR) (Figure C) (Channon, 2004). BH4 is susceptible to oxidation by reactive oxygen species such as $ONOO^-$, forming dihydrobiopterin (BH2) and ultimately biopterin (Channon, 2004). Synthesis of BH4 is also possible through the salvage pathway from the synthetic pterin, sepiaterin, which is metabolized to BH2 by SR and then to BH4 by DHFR (Figure C) (Channon, 2004).

GTP-CH 1 is regulated by transcriptional induction in response to stimuli such as cytokines (Hattori et al., 1997). However, inflammatory stimuli do not appear to greatly up-regulate vascular GTP-CH 1 expression in vivo (Alp et al., 2003). Posttranslational modification of GTP-CH 1 activity by phosphorylation and by feedback inhibition through protein-protein interactions may be important additional regulators of BH4 synthesis (Thony et al., 2000). Furthermore, GTP-CH 1 feedback regulatory protein expression in endothelium is reduced in response to cytokines, resulting in increased BH4 synthesis independent of GTP-CH 1 expression (Werner et al., 2002).

For salvage pathway, there is a limited evidence for DHFR in an in vivo study. It seems to be that BH2 is the preferential form in the cells since BH4 once taken up from the extracellular fluid was immediately oxidized to BH2 (Yamamoto et al., 1997). Moreover, BH4 administered to individual animals was also oxidized to BH2 (Hasegawa et al., 2005). The animals supplemented with BH4 could not increase tissue BH4 level when treated by an inhibitor of DHFR (Sawabe et al. 2004). This implies that DHFR plays a critical role in generation of BH4 via the salvage pathway.

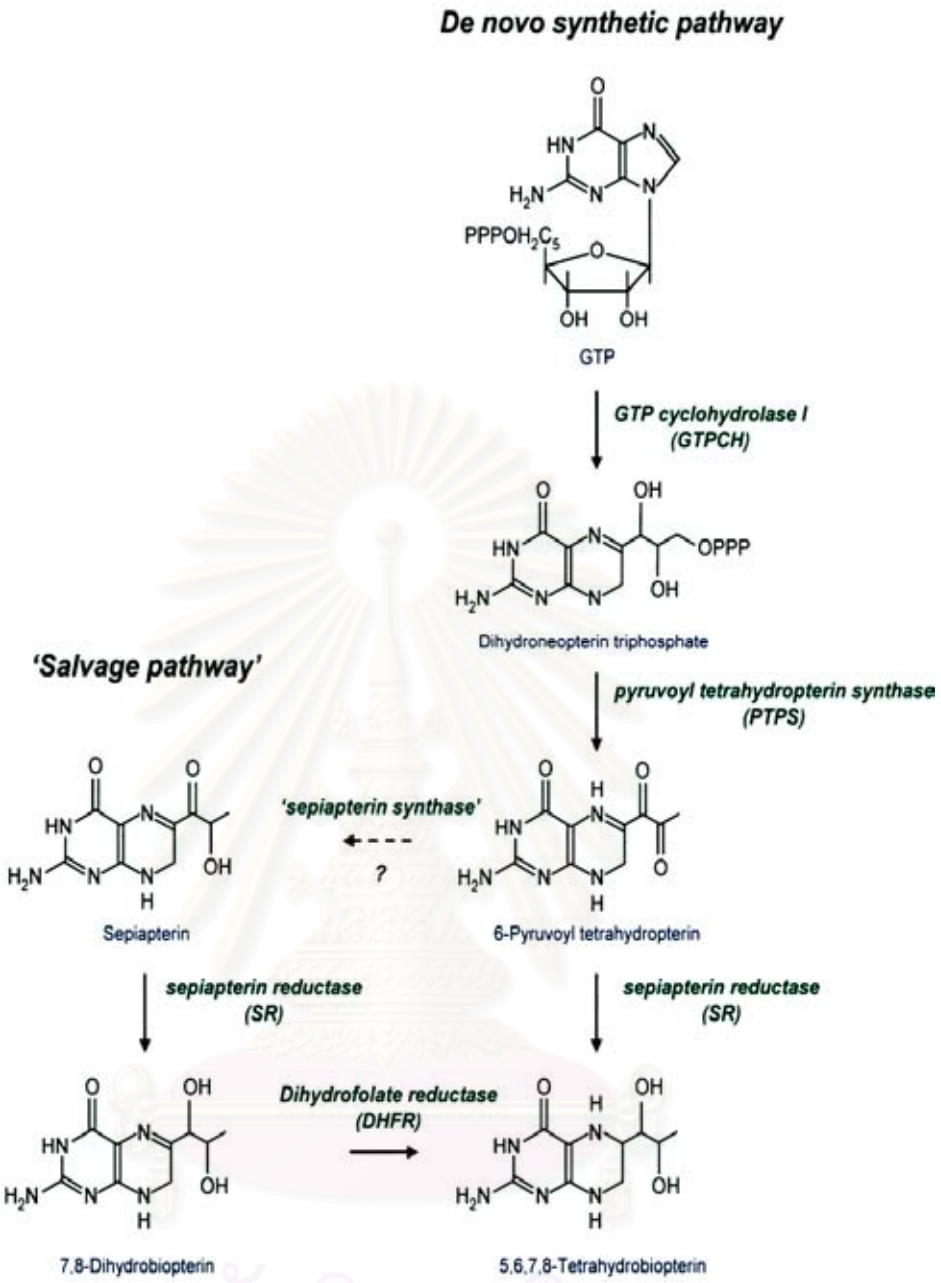


Figure C: Tetrahydrobiopterin (BH4) biosynthesis and metabolism.

More recent studies have demonstrated the implication of ANG II on DHFR. For instance, ANG II administration (300 ng/kg/min, subcutaneously, osmotic pump) could activate iNOS, NO, $O_2^{\cdot}$, and $ONOO^-$ generation which are reversed by BH4 administration (Kase et al., 2005). The similar results also occur in renal IR condition that ANG II is induced. Kakoki et al. (2000) have demonstrated that BH4

supplementation in IR rats reduces NOS uncoupling as well as increases NO generation and eNOS dimerization. Furthermore, the unveiled action of ANG II on DHFR has been elucidated by Chalupsky and Cai (2005). They have demonstrated that ANG II rapidly and transiently activates NADPH oxidase to produce $O_2^{\cdot}$, leading to H_2O_2-dependent down-regulation of DHFR in BAEC. In addition, a second-phase $O_2^{\cdot}$ production from uncoupled NOS has also been observed (Chalupsky and Cai 2005). The overexpression of DHFR could restore NO production and abolish the changes induced by ANG II (Chalupsky and Cai, 2005). These data demonstrate an essential role of DHFR in maintaining BH4 and NO bioavailability, and ANG II could modify DHFR under these circumstances. However, the exogenous H_2O_2 could up-regulate GTP-CH 1 expression which increases total biopterin, but the endogenous H_2O_2 produced by ANG II does not enhance GTP-CH 1 (Chalupsky and Cai, 2005; Shimizu et al., 2003). These results suggest that DHFR is much more sensitive to lower endogenous H_2O_2 compared to GTP-CH 1, which is regulated by exogenous H_2O_2.

Since renal IR causes an increase in ANG II level, it could be postulated that ANG II would affect NOS expression by modulating BH4 synthesis via alteration in DHFR or GTP-CH 1, or both. However, to date, there is no study on IR in relation to renal DHFR, GTP-CH 1, and NOS expression as well as the role of angiotensin and angiotensin receptor type 1 in this condition. To accomplish these aims, ACEI and ARB were used in the present experiment.

CHAPTER III

MATERIALS AND METHODS

EXPERIMENTAL ANIMALS

The study was performed in male Wistar rats weighing 220 to 250 grams obtained from the National Center of Scientific Use of Animals (Mahidol University, Salaya, Nakhonpathom). The rats were housed in a well ventilated room in which the temperature was 23 to 25°C with an automatic lighting schedule, which provided darkness from 8 p.m. to 6 a.m. The animals were given free access to standard laboratory chow and water as follow the experimental protocol. All rats were used once only. At the end of each experiment, the rats will be terminated with an overdose of sodium pentobarbital by intraperitoneal injection and their remains will be eliminated by burning in an incinerator.

CHEMICALS

Chemical agents

Sodium pentobarbital (Nembutal®) was purchased from Sanofi, France. Enalapril® (20 mg) was obtained from Biolab, Thailand. Losartan® (50 mg) was obtained from M&H, USA. TRIzol® reagent was purchased from Invitrogen, USA. Deoxynucleotide triphosphates (dNTPs), Ribonuclease inhibitor, Maloney murine leukemia virus (M-MLV) reverse transcriptase, Taq DNA Polymerase, 100 bp DNA ladder marker, and agarose LE analytical grade were purchased from Promega, USA. Protease inhibitor cocktail was purchased from Sigma, USA. Modified Lowey protein

assay kit and SuperSignal® West Pico kit were purchased from Pierce, USA. Prestained protein molecular marker, Laemmli sample buffer, ammonium persulfate, 40% acrylamide/Bis, glycine, TEMED, methanol, sucrose, filter paper, nitrocellulose membrane, and CL-XPosure™ Film 5 x 7 inches were purchased from Pierce, USA.

Antibodies

Monoclonal anti-dihydrofolate reductase, N-terminal, developed in rabbit (DHFR, Catalog No. D1067), mouse monoclonal antibody against NOS II (inducible NOS, Catalog No. N39020-150), and NOS III (endothelial NOS, Catalog No. N30020-150) were purchased from Sigma, USA. GTP-cyclohydrolase 1 monoclonal antibody (Catalog No. H00002643-M01) was purchased from Abnova Corporation, Taiwan. β-Actin mouse monoclonal antibody (Catalog No. A5441, Sigma, USA) was kindly provided by Assistant Professor Sarinee Kalandakanond-Tongsong, DVM, Ph.D, Faculty of Veterinary, Chulalongkorn University. Blotting grade goat anti-rabbit IgG (H+L) (Human IgG Adsorbed) horseradish peroxidase conjugate (Catalog No. 170-6515) and blotting grade goat anti-mouse IgG (H+L) (Human IgG Adsorbed) horseradish peroxidase conjugate (Catalog No. 170-6516) were purchased from Bio-Rad, USA.

EXPERIMENTAL PROCEDURE

The animals were divided into two main groups sham (S) (n = 24) and IR (n = 24). Each group was further divided into three subgroups, which treated with three different kinds of drinking solution as follow:

1. Water (only distilled water)
2. Water + angiotensin converting enzyme inhibitor, ACEI

   (Enalapril® 5 mg/kg/day)
3. Water + angiotensin type 1 receptor blocker, ARB

   (Losartan® 10 mg/kg/day)

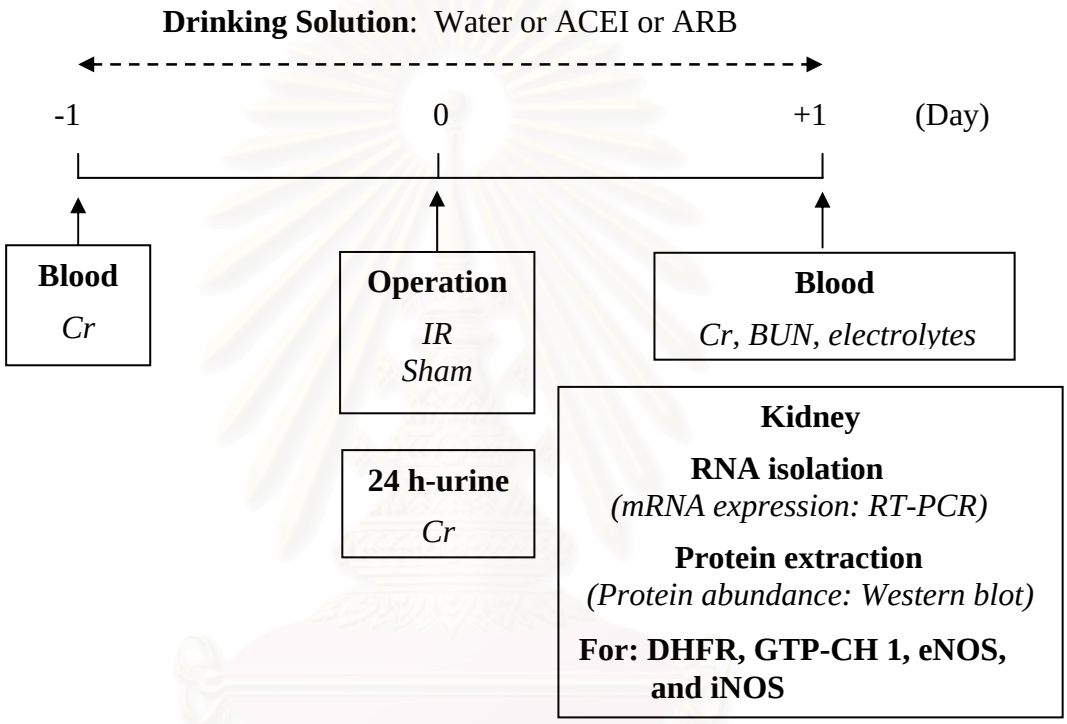
Surgical Operation

After three days of keeping to familiar with the new housing, the rats were weighed and blood samples were collected from the tail for measuring creatinine (Cr) in order to assess kidney function (less than 1 mg %). The rats were anesthetized by intraperitoneal (i.p.) injection of sodium pentobarbital (60 mg/kg body weight). Using aseptic technique, a midabdominal incision was made to expose the kidneys. For IR groups, a unilateral left renal pedicle cross clamp was performed for 30 minutes with microvascular clamps and then was released for reperfusion. The incision was closed. For sham (S) groups, the rats were operated and only wiped left renal pedicle. Each rat was allowed to wake up and return to a metabolic cage with free access to food but with three different kinds of drinking solution as described above.

The volume of drinking solution was approximately 30 ml/rat/day. ACEI or ARB was given one day before the IR or S operation and continuously for 1 day after the operation (n = 8 rats/group). Twenty four-hour urine was collected before the due date. On experimental due date, the rats of respective groups were re-operated. Blood samples were collected from the aorta and centrifuged at 3500 rpm (H-103N, Kokusan, Tokyo, Japan). Serum samples were stored at -80°C until use for blood urea nitrogen (BUN), Cr, and electrolytes measurements by using ISE (ion

selection electrode) indirect method (Model CX3, Beckman, INC, Germany). The kidneys were removed and fixed in liquid nitrogen, and then stored at -80°C until use for RNA isolation and protein extraction.

EXPERIMENTAL DESIGN



DETERMINATION OF mRNA EXPRESSION BY REVERSE TRANSCRIPTION POLYMERASE CHAIN REACTION (RT-PCR)

Principal: RT-PCR is a method used to amplify cDNA copies of RNA. With sensitive and versatile, RT-PCR is used to retrieve and clone the 5´ and 3´ termini of mRNAs and to generate large cDNA libraries from very small amounts of mRNA (Framer and Coen, 2001). In addition, RT-PCR can be easily adapted to identify mutations and polymorphisms in transcribed sequences and to measure the strength of

gene expression when the amounts of available mRNA are limited and/or when the RNA of interest is expressed at very low levels.

The literature is abundantly adorned with descriptions of variants of RT-PCR, many of which have their own acronym. The technical details may vary from one paper to the next, but the underlying concepts are constant and relatively simple. In every case, the first step is the enzymatic conversion of RNA to a single-stranded cDNA template. An oligodeoxynucleotide primer is hybridized to the mRNA and is then extended by an RNA-dependent DNA polymerase to create a cDNA copy that can be amplified by PCR (Framer and Coen, 2001). Depending on the purpose of the experiment, the primer for first-strand cDNA synthesis can be specifically designed to hybridize to a particular target gene or it can bind generally to all mRNAs.

Total RNA Isolation by Using Trizol® Reagent

Principal: Total RNA was extracted from the frozen left kidney by using Trizol reagent (Invitrogen, USA). Trizol reagent (cleared-red color) is a ready-to-use reagent for the isolation of total RNA from cells and tissues. The reagent, a mono-phasic solution of phenol and guanidine isothiocyanate, is an improvement to the single-step RNA isolation method developed by Chomczynski and Sacchi (1987). During sample homogenization or lysis, Trizol reagent will disrupt cells and dissolve cell components but the reagent still maintains the integrity of the RNA. The RNA isolation procedure is performed in the sequential steps as follow:

Homogenization

The tissue samples were homogenized in Trizol reagent (1 ml/ 50-100 mg of tissue) by using a polypropylene micropestle (Eppendorf, USA). The sample volume should not exceed 10% of the volume of Trizol reagent used for

homogenization. To remove insoluble material, the homogenate was centrifugated at 12,000xg for 10 minutes at 2 to 8°C (Biofuge Primo R Heracus, Germany). The resulting pellet contains extracellular membranes, polysaccharides, and high molecular weight DNA, while the supernatant contains total RNA. In each sample, the supernatant was transferred to a fresh tube.

Phase separation

To permit the complete dissociation of nucleoprotein complexes, the supernatant samples were added with 0.2 ml of chloroform per 1 ml of Trizol reagent. The tubes were capped securely and shaken vigorously by hand for 15 seconds and incubated them at room temperature (15-30°C) for 2 to 3 minutes. Centrifuge the samples at 12,000xg for 15 minutes at 4°C. Following centrifugation, the mixture separates into a lower red, phenol-chloroform phase, an interphase, and a colorless upper aqueous phase. The RNA remains exclusively in the aqueous phase which is approximately 60% of the volume of Trizol reagent used for homogenization.

RNA precipitation

The aqueous phase was transferred to a fresh tube, and then precipitated by mixing with isopropyl alcohol (0.5 ml/ 1 ml Trizol reagent volume). The samples were allowed to sit for 10 minutes at room temperature and centrifuged at 12,000xg for 10 minutes (4°C). The precipitation of RNA will form a gel-like pellet on the side and bottom of the tube.

RNA wash

The supernatant was removed. The RNA pellet was washed once with 75% ethanol (1 ml/ 1 ml of Trizol reagent used for the initial

homogenization). The pellet was mixed by vortex and then centrifuged at 7,500xg for 5 minutes at 4 °C.

Re-dissolving the RNA

Finally, the RNA pellet was dry briefly (air-dry or vacuum-dry for 5-10 minutes; not by centrifugation under vacuum). It is important not to let the RNA pellet dry completely as this will greatly decrease its solubility. To dissolve RNA, RNase-free water was added into the tube and mixed by passing the solution a few times through a pipette tip. Then, the dissolved RNA solution was incubated for 10 minutes at 55 to 60°C. A portion of dissolved RNA sample was measured the absorbance which should have a ratio of 260/280 at 1.6 to 1.8. The samples were then stored at -80°C until use for cDNA synthesis.

Reverse Transcription (cDNA Synthesis)

To synthesize cDNA, a volume of 1 μg of total RNA was reverse transcribed to a first-strand cDNA in reverse transcription buffer containing 5x reaction buffer, 0.8 mM deoxynucleotide triphosphate (dNTP) mixture, reverse primer of target and house keeping gene glyceroldehyde-3-phosphate-dehydrogenase (GAPDH), 1U/μl Ribonuclease inhibitor, 0.25 U/μl Maloney Murine Leukemia Virus (M-MLV) reverse transcriptase and RNase free water (Promega, USA). The mixture samples were then incubated at 45˚C for 45 min and inactivated at 99˚C for 5 min by using thermocycler (Perkin Elmer, USA). Then the samples were kept at 4˚C until use for amplification.

Polymerase Chain Reaction (cDNA Amplification)

To amplify cDNA, the PCR reaction was performed in a total volume of 50 µl. A 5 µl of cDNA was used as templates for subsequent PCR reaction in the thermocycler. The PCR reaction was composed of distilled water, 10x PCR buffer, 2 mM $MgCl_2$, 200 µM dNTP mixture, 0.04 U/µl Taq DNA polymerase (Promega, USA) and specific oligonucleotide primers (sense and antisense) derived from rat cDNA sequence (www.ncbi.nlm.nih.gov). The primers were designed by using Primer3 Input program (www.cgi v0.2) with base pair length as shown in Table A. The PCR amplification was performed by using PCR thermal programs for 35 cycles under the following condition: denaturation at 94˚C for 1 min, annealing for 1 min at different temperatures (Table A) and extension for 90 sec at 72˚C. To ensure that equal amounts of cDNA were added to PCR mixture, GAPDH (515 bp) Sense 5′-AATGCATCCTGCACCACCAA-3′, and Antisense 5′-GTAGCCATATTCATTGT CATA-3′ were also subjected to amplify in the same reaction of each primer pair. In addition, to comfirm that there was no genomic DNA contamination in the RNA samples, the PCR reaction was performed without reverse transcription. As a control reaction, the RNase free water was replaced the RNA samples in the reaction.

Table A: Characteristics of the primers that were used for RT-PCR analysis of DHFR, GTP-CH1, eNOS, and iNOS genes in sham and IR rat [a]

Genes Targeted (Accession No.)	Primer Sequences	Product Size (bp)	Annealing Conditions
DHFR (NM_ 130400)	5′-ACCAGGAAGCCATGAATCAG-3′ (S) 5′-AGCAGTAGGACTTGGGAGCA-3′ (AS)	224	56˚C, 1 min
GTP-CH 1 (NM_024356)	5′-GGGAAGGGTCCATATTGGTT-3′ (S) 5′-ACCTCGCATGACCATACACA-3′ (AS)	205	55˚C, 1 min
eNOS (NM_021838)	5′-TGACCCTCACCGATACAACA-3′ (S) 5′-CTGGCCTTCTGCTCATTTTC-3′ (AS)	210	56˚C, 1 min
iNOS (U_03699)	5′-GTGTTCCACCAGGAGATGTTG-3′ (S) 5′-GAAGGCGTAGCTGAACAAGG-3′ (AS)	272	60˚C, 1 min

[a] RT-PCR, reverse transcriptase-PCR; DHFR, dihydrofolate reductase; GTP-CH 1, GTP-cyclohydrolase1; eNOS, endothelial NOS; iNOS, inducible NOS; bp, base pair; S, sense primer; AS, antisense primer.

Gel Electrophroresis and Image Analysis

Subsequently, the amplified PCR products were subjected to gel electrophoresis (Bio-Rad, USA), and visualized by High Resolution UV and White Light Gel Doc System (Bio-Rad, USA). Briefly, a 10 µl of each PCR product was mixed with 2 µl of loading buffer and then loaded into 1.5% agarose gel containing ethidium bromide. The gel was run at 100 volts (Bio-Rad, USA) until front dye reached the end of gel. Estimated size of PCR products was compared to the standard 100 bp ladder marker (Promega, USA). The intensity of the product bands was quantified by High Resolution UV and White Light Gel Doc System linked to a computer analysis system (Quantity One User Guide for Version 4.2; Bio-Rad, USA). The intensity ratio of each studied gene to GAPDH was calculated.

DETERMINATION OF PROTEIN ABUNDANCE BY USING WESTERN BLOT ANALYSIS

Principal: Immuno-blotting is a widely used and powerful technique for the detection and identification of protein using antibodies. The process involves the separation of sample proteins by polyacrylamide gel electrophroresis (PAGE) followed by transferring of the separated proteins from the gel onto a thin support membrane. The membrane binds and immobilizes the proteins in the same pattern as in the original gel. The membrane (or “blot”) is then exposed to a solution containing antibodies that recognize and bind to the specific protein of interest. The antibodies bound to the membrane are detected by any of a variety of techniques, usually involving treatment with a secondary antibody.

Separation of Protein by

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Principal: The most widely used method for qualitative analysis of a protein mixture is SDS-PAGE by using the buffer system of Laemmli (1970). With this method, it is possible to determine both the purity and the relative molecular weight of an unknown isolated protein or proteins. In the process, proteins migrate in response to an electrical field through pores in the gel matrix and separate based on molecular size after sample proteins are solubilized by boiling at 100˚C, 5 minutes in the presence of anionic detergent and 2-Mercaptoethanol (2-ME). The 2-ME is a disulfide reducing agent, and serves to reduce disulfides holding together the tertiary structure of the protein. The anionic SDS detergent binds strongly to the protein thus disrupting its secondary, tertiary and quaternary structure, resulting in a linear polypeptidles chain coated with negatively charged SDS molecules. The binding efficiency of the SDS is generally one SDS molecule for every two amino acid residues. Under this condition, the polypeptide chains are unfold and assumed a rod-like structure and have negative charge, resulting in a constant charge to mass ratio. Then proteins move through a polyacrylamide gel matrix toward the anode. The polyacrylamide gel is cast as a separating gel topped by a stacking gel and secured in an electrophoresis apparatus. Separation is determined by size and therefore when compared to standards of known molecular weight, the relative molecular mass can be estimated. Purity is determined by the presence of a band associated with the desired protein and the absence of bands associated with contaminating proteins.

The SDS gel is comprised of a main separating gel and a stacking gel. The proteins, which have been mixed with a loading buffer, containing on ionizable tracking dye bromophenol blue, are loaded into wells formed in the stacking gel. A

current is passed through the gels and the proteins migrate through the stacking gel and are concentrated into a solid band at the separating gel. When the proteins enter into the separating gel, the negatively charged protein-SDS complexes migrate toward the anode. Their migration in the gel relative to each other is the same based on their uniform negative charge. Separation, therefore, occurs as a result of the molecular sieving properties of the gel. The larger the protein, the more its mobility is retarded by the frictional resistance of the gel the smaller the molecule the further its mobility in the gel. The bromophenol blue is completely unretarded in the gel due to its small size relative to proteins and it is thus used to monitor the progress of the electrophoresis. The current is turned off once the tracking dye has migrated to bottom of the gel. The experimental protocol is performed by the sequential steps as follow:

Assembly of apparatus

The reagents used in preparing the gel should be removed from the refrigerator and allowed to warm and degas for one hour prior to the preparation of the gel. Meanwhile, the sandwich plates were set up for casting the gel. A sandwich consists of two rectangular glass plates: the outer is 10.1 cm (width) x 8.3 cm (height) and the inner is 10.1 cm (width) x 7.3 cm (height) separated by spacers of 0.75 mm thickness (Mini-Protein® 3 cell, Bio Rad, Canada). In order to prepare a flawless gel, one containing has no air bubble or debris, the glass plates must be perfectly cleaned and dry with absolute ethanol. The casting clamps were used to mount the outer and inner glass plates facing together.

Preparation of slab gel

For separating gel, 10% or 7.5% acrylamide was carefully filled into the space of sandwich plates from bottom to top with no air bubbles. The height of the gel was adjusted by the comb, approximately 1 cm below the bottom edge. The top layer was filled with 1 ml of distilled water. The gel was allowed to polymerize at room temperature for 30 min. After polymerization, the water was drained off and excess liquid was removed with a piece of Whatman 3 MM paper. The selected comb (number of wells, thickness same as spacers) was gently inserted, then 4% acrylamide solution was filled into the space for making the stacking gel. It should be made sure that no air bubbles formed around the teeth the comb, as they will impede the migration and separation of the proteins. The gel was allowed to polymerize for one hour at room temperature.

Preparation of sample

During polymerizing the stacking gel, equal amounts of total protein from each sample were mixed with sample buffer. The mixers were heat for 5 min at 100 ºC in a boiling water bath (Thermostatic bath/circulator; Grant W6, UK). To detect NOS dimmer form, the heating step was omitted. The amount of total protein used for total NOS was 50 μg, and for DHFR, GTP-CH1, monomer or dimer of NOS was 100 μg.

Electrophoresis

After polymerization is complete, the comb was gently removed. The wells were filled with running buffer (Appendix). The gel was mounted in the electrophoresis apparatus and the upper buffer chamber was filled with running buffer. Remove any air bubbles trapped at the bottom of the wells. This will disrupt

the electrical circuit and an uneven electrophoresis. Each protein sample was loaded into the bottom of each well. The molecular weight markers (Fermentus, USA) were also loaded. Then, the running buffer was poured into the lower chamber. The electrophoresis apparatus was attached to an electric power supply (PowerPac^{TM} HC, Bio-Rad, USA) and turned on at 125 volt. Small bubbles should start to be produced and rise off the electrode wire at the bottom of the lower chamber. If no bubbles appear then there is an electrical circuit problem. The gel was run until the dye front reaches the bottom of gel. To detect dimer form of NOS the temperature of the gel was maintained below 15°C during electrophroresis (low temperature-PAGE, LT-PAGE). The power supply was turned off. Then, the gel from the glass plate was removed and placed into the transfer buffer.

Protein transfer

A nitrocellulose membrane (Trans-Blot®, Bio-Rad, USA) and two sheets of absorbent filter paper were cut into the same size of the gel. The membrane, filter papers, and support pads were soaked in the transfer buffer for 10 min. The transfer cassette was assembled by lying the black side down, then plate a support pad, filter paper, gel, membrane, filter paper, and support pad. Lock and put the complete transfer cassette into the transfer tank containing transfer buffer by facing the membrane side to the positive electrode (anode, red electrode) and transferred for 120 min at 100 volt. After transfer, the membrane was removed from the cassette and immersed into the blocking solution.

Blocking

The membrane was incubated in blocking solution (5% non fat dry milk in PBS-0.1% Tween) for 1 h at room temperature with gentle rotation on a

platform rocker (Orbital Shaker, UK). This process will reduce the background of non-specific binding site with irrelevant protein. After blocking, the membrane was washed for 10 min 2 times by PBS-0.1% Tween washing buffer.

Detection of bound antibody

The dilution of primary antibody was prepared in PBS-0.1% Tween (1:1000 for DHFR, GTP-CH 1, and iNOS; 1: 500 for eNOS and 1:100000 for β-actin). The membrane, in a clean plastic box, was incubated with the respective primary antibody overnight at 4°C on a platform rocker. After incubation, the primary antibody solution was discarded. The membrane was washed with 20 ml of PBS for 10 min 2 times. The secondary antibody was prepared in PBS-0.1% Tween. The goat anti rabbit IgG conjugated HRP antibody at 1:20,000 dilution was used for DHFR. The goat anti mouse IgG conjugated HRP antibody at 1:20,000 dilution was used for GTP-CH 1, iNOS, and β-actin, and at 1: 5,000 dilution for eNOS. The membrane was incubated in the secondary antibody for 1 h at room temperature on a platform rocker. After that, the blotting membrane was washed with PBS-0.1% Tween for 10 min 2 times on a platform rocker.

Protein detection and image analysis

The blotting membrane was placed on a cleaned glass plate. The detection reagent (SuperSignal® West Pico kit, Pierce, USA) was prepared by mixing equal parts of the stable peroxide solution and the luminal/enhancer solution, and then overlay the reagent directly on the membrane surface carrying the protein. After incubation for one min. at room temperature, the excess reagent was drained off, and wrapped by a piece of saran wrap. It is necessary to work quickly once the membrane has been exposed to the detection solution. In a dark room, the membrane

was placed, protein side up, in a X-ray film cassette. The lights were turned off and a sheet autoradiography film (CL-XPosure™ Film 5 x 7 inches, Pierce, USA) was carefully placed on the top of the membrane, then the cassette was closed and exposed for a certain period, eg. 1 min (this depends on the amount of target protein on the membrane). The film was developed by X-Ray film processor (Optimax® 2010, Germany) and scanned the intensity by using high resolution scanner (Image class MPC190, Canon H12260, Canada). Each intensity band was quantified by High Resolution UV and White Light Gel Doc System linked to a computer analysis system (Quantity One User Guide for Version 4.2; Bio-Rad, USA). The intensity ratio of each studied protein to β-actin was calculated.

DETERMINATION OF PROTEIN CONCENTRATION BY LOWRY METHOD

Protein Extraction

Each frozen renal tissue was homogenized on ice with a homogenizer (T25 Basic, IKA, Malaysia) in homogenization buffer (20 mM Tris-HCl; pH 7.5, 2mM $MgCl_2$, 0.2 M sucrose, and 5% v/v protease inhibitor cocktail, Sigma, USA). The homogenates were centrifuged at 12,000 g for 20 min at 4˚C. The resulting pellet containing cell nuclei and tissue debris was discarded. The protein concentration of the supernatant was collected and aliquot into a sterile microcentrifuge tube, kept at -80˚C until use. All samples will be further assayed for the amount of protein by using a modified Lowry assay kit (Bio-Rad, USA).

Protein Assay

Principal: For many years, Lowry's method was the most widely used and cited procedure for protein quantitation (Lowry et al., 1951). The procedure involves the reaction of protein with cupric sulfate and tartrate in an alkaline solution, resulting in formation of tetradentate copper-protein complexes. When the Folin-Ciocalteu reagent is added, it is effectively reduced in proportion to these chelated copper complexes, producing a water-soluble product whose blue color can be measured at 750 nm. The protocol is performed by the sequential step as follow:

Preparation of serum bovine albumin (BSA) standards

Five standard dilutions in duplicate (0, 50, 100, 200, and 500 μg/ml) were prepared by dissolving the stock standard BSA solution (2 mg/ml) as indicated in Table B. Pipette double distilled deionized water 180, 450, 250, 250 and 200 μl into tube No.1-5, respectively. Then pipette BSA stock (2mg/ml) 60 μl into tube No.1 and 50 μl into tube No.2, mixed each tube thoroughly. Then, pipette 250 μl of solution from tube No.2 into tube No.3 and mixed well. Finally, pipette 250 μl of solution from tube No.3 into tube No.4, then mixed each tube thoroughly. There will be sufficient volume of each diluted standard for two replications.

Table B: Preparation of serum bovine albumin (BSA) standards

Tube Number	BSA (μg/ml)	BSA (μl)	DDW (μl)	Total volume (μl)
1	500	60	180	240
2	200	50	450	500
3	100	250	250	500
4	50	250	250	500
5	0	-	200	200

Note: DDW = Double distilled deionized water

Preparation of 1X Folin-Ciocalteu reagent

Prepare 1X (1 N) Folin-Ciocalteu reagent by diluting the supplied 2X (2 N) reagent 1:1 with ultrapure water. Because the diluted reagent is unstable, prepare only as much 1X Folin-Ciocalteu reagent as will be used in one day. Each test replicate requires 50 μl of 1X Folin-Ciocalteu reagent in the test tube protocol.

Procedure to Quantitate Total Protein

1. Pipette 0.1 ml of each standard and unknown sample replicate into an appropriately labeled test tube.
2. At 15-sec intervals, add 0.5 ml of Modified Lowry Reagent to each test tube. Mix well and incubate each tube at room temperature for exactly 10 min.

3. Exactly at the end of each tube's 10-min incubation period, add 50 µl of prepared 1X Folin-Ciocalteu Reagent, immediately vortex to mix the contents. Maintain the 15-sec interval between tubes established in step 2.
4. Cover and incubate all tubes at room temperature for 30 min.
5. With the spectrophotometer set to 750 nm, zero the instrument by a cuvette filled only with water. Subsequently, measure the absorbance of all the samples.
6. Subtract the average 750 nm absorbance values of the blank standard replicates from the 750 nm absorbance values of all other individual standard and unknown sample replicates.

CALCULATION FOR ASSESSMENT OF RENAL FUNCTION

$$\text{Creatinine clearance } (C_{Cr}) = \frac{U_{Cr} \times V}{P_{Cr}}$$

STATISTICAL ANALYSIS

All data were expressed as mean ± SD. For comparison among groups of animals, one way analysis of variance (one-way ANOVA) was used and the differences in pairs of means among groups were made by Tukey's test. If the statistical probability (p-value) was less than 0.05, the differences were considered to be statistically significant.

CHAPTER IV

RESULTS

Effects of ACEI or ARB on renal DHFR and GTP-CH 1 mRNA expression in S or IR

As shown in Figure 1, in S or S treated groups (with ACEI or ARB), the renal DHFR mRNA expression was comparable (S = 100 %, S + ACEI = 104.79 $\pm$ 12.02 %, S + ARB = 103.47 $\pm$ 15.36 %). IR significantly decreased the expression to be 66.74 $\pm$ 14.46 % ($p<0.01$ as compared with S or S treated groups). Treatment with ACEI or ARB could restore the DHFR mRNA levels to be 93.54 $\pm$ 10.04 % ($p< 0.05$) and 101.52 $\pm$ 17.21 % ($p<0.01$), respectively.

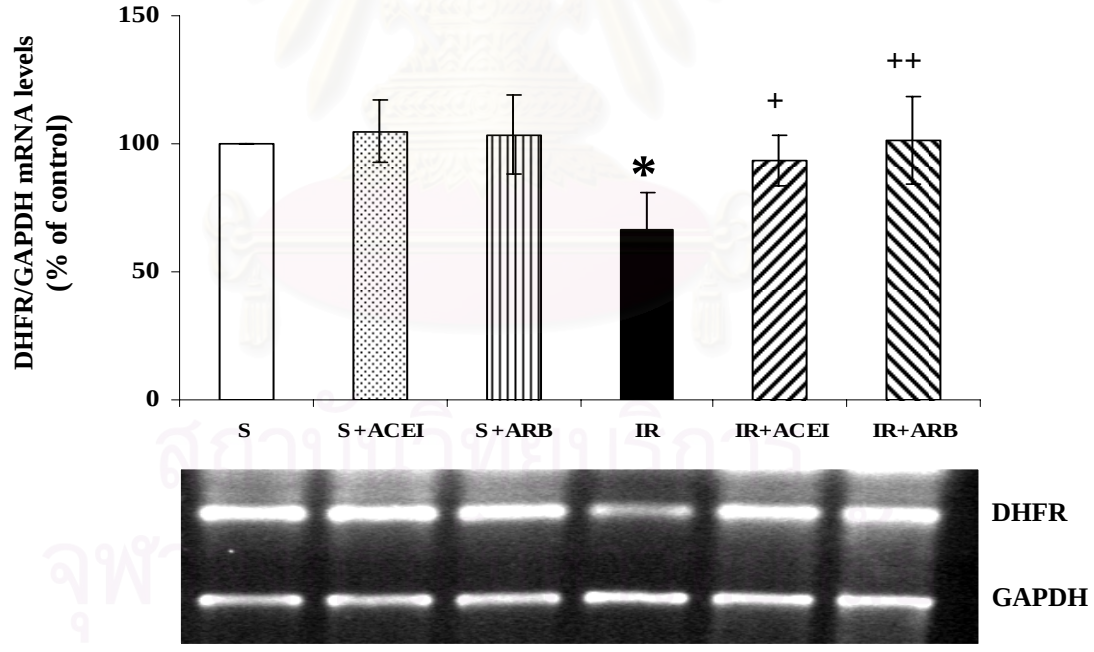


Figure 1: Effects of ACEI or ARB on renal DHFR mRNA expression in S or IR rats (n = 8/group). The upper panel is histogram bars showing the ratio of DHFR to GAPDH intensity. The lower panel is a representative ethidium bromide-stained agarose gel photograph for 515 bp GAPDH mRNA and 224 bp DHFR mRNA. * $p<0.01$ vs S or S treated groups, + $p<0.05$, ++ $p<0.01$ vs IR

For GTP-CH1 (Figure 2), either IR or the inhibition of ANG II had no significant effect on the mRNA levels. The expression was comparable in all groups studied (S = 100 %, S + ACEI = 100.01 ± 9.96 %, S + ARB = 98.33 ± 11.11 %; IR = 101.23 ± 12.81 %, IR + ACEI = 96.54 ± 11.18 %, IR + ARB = 97.54 ± 12.37%).

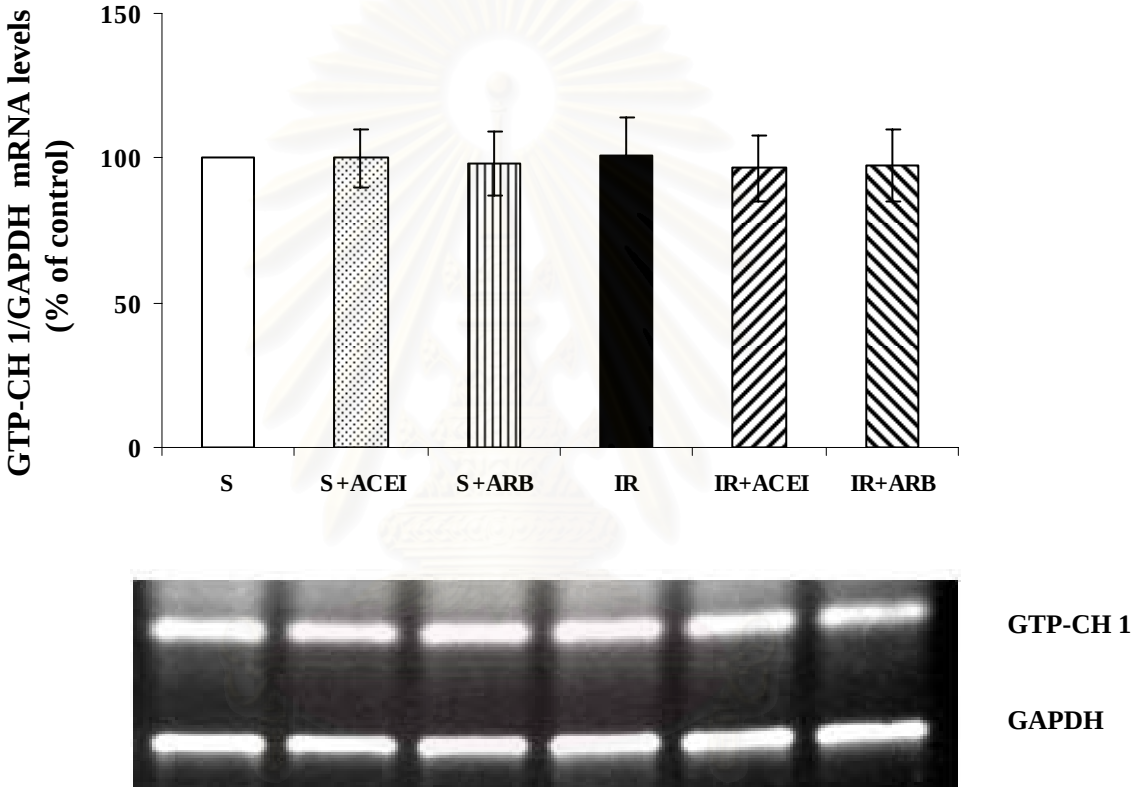


Figure 2: Effects of ACEI or ARB on renal GTP-CH 1 mRNA expression in S or IR rats (n = 8/group). The upper panel is histogram bars showing the ratio of GTP-CH 1 to GAPDH intensity. The lower panel is a representative ethidium bromide-stained agarose gel photograph for 515 bp GAPDH mRNA and 205 bp GTP-CH 1 mRNA.

Effects of ACEI or ARB on renal DHFR and GTP-CH 1 protein abundance in S or IR

As illustrated in Figure 3, IR significantly decreased renal DHFR protein to be 79.57 ± 7.70 % ($p<0.01$) as compared with S or S treated groups (S = 100 %, S + ACEI = 102.04 ± 6.53 %, and S + ARB = 105.41 ± 5.46 %). Treatment with ACEI or ARB could restore DHFR protein levels to be 95.31 ± 6.59 % and 94.95 ± 6.50 % ($p<0.01$), respectively.

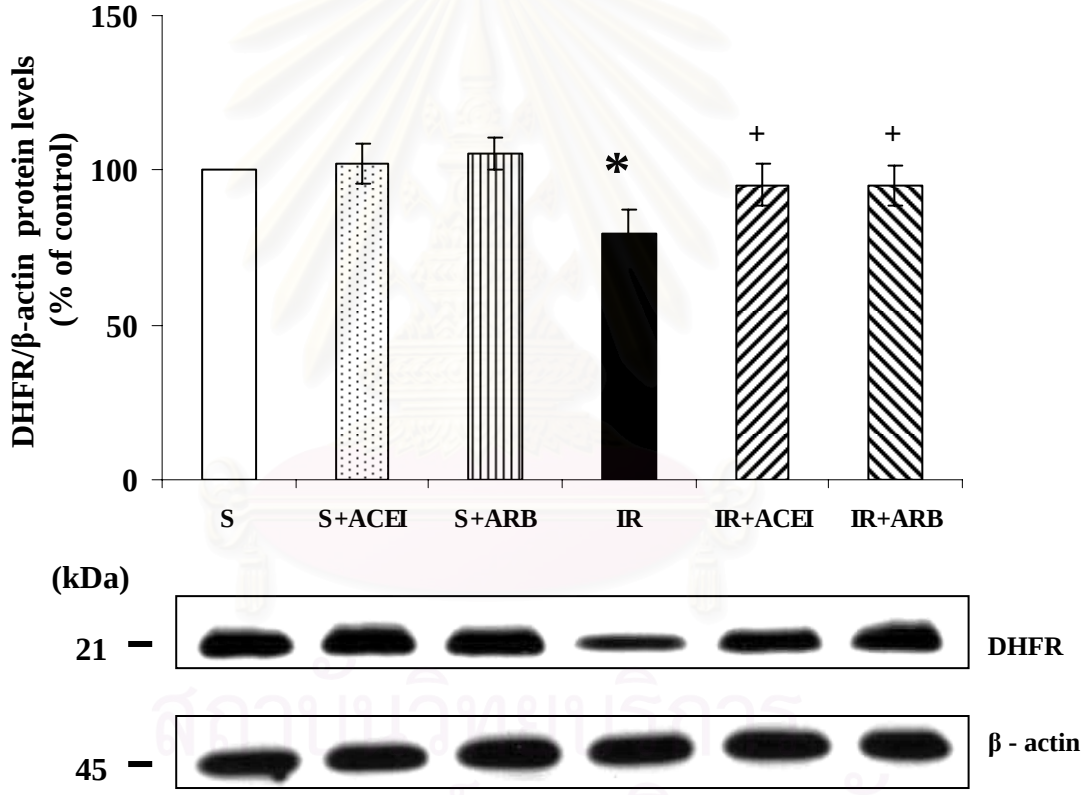


Figure 3: Effects of ACEI or ARB on renal DHFR protein abundance in S or IR rats The upper panel is histogram bars showing the results of densitometric analyses from pooled data (n = 8/group). Values were normalized by the corresponding optical density for β-actin, used as internal control. The lower panel shows the results of immunoblots analyzed in renal tissue, and probed with antibodies against DHFR (21 kDa) or β-actin (45 kDa). * $p<0.01$ vs S or S treated groups, + $p<0.01$vs IR

For GTP-CH1 (Figure 4), either IR or the inhibition of ANG II had no significant effect on the protein levels. The abundance was comparable in all experimental groups (S = 100 %, S + ACEI = 100.80 ± 9.20 %, S + ARB = 103.98 ± 9.29 %, IR = 106.35 ± 7.35 %, IR + ACEI = 102.45 ± 9.58 %, IR + ARB = 103.06 ± 11.41%).

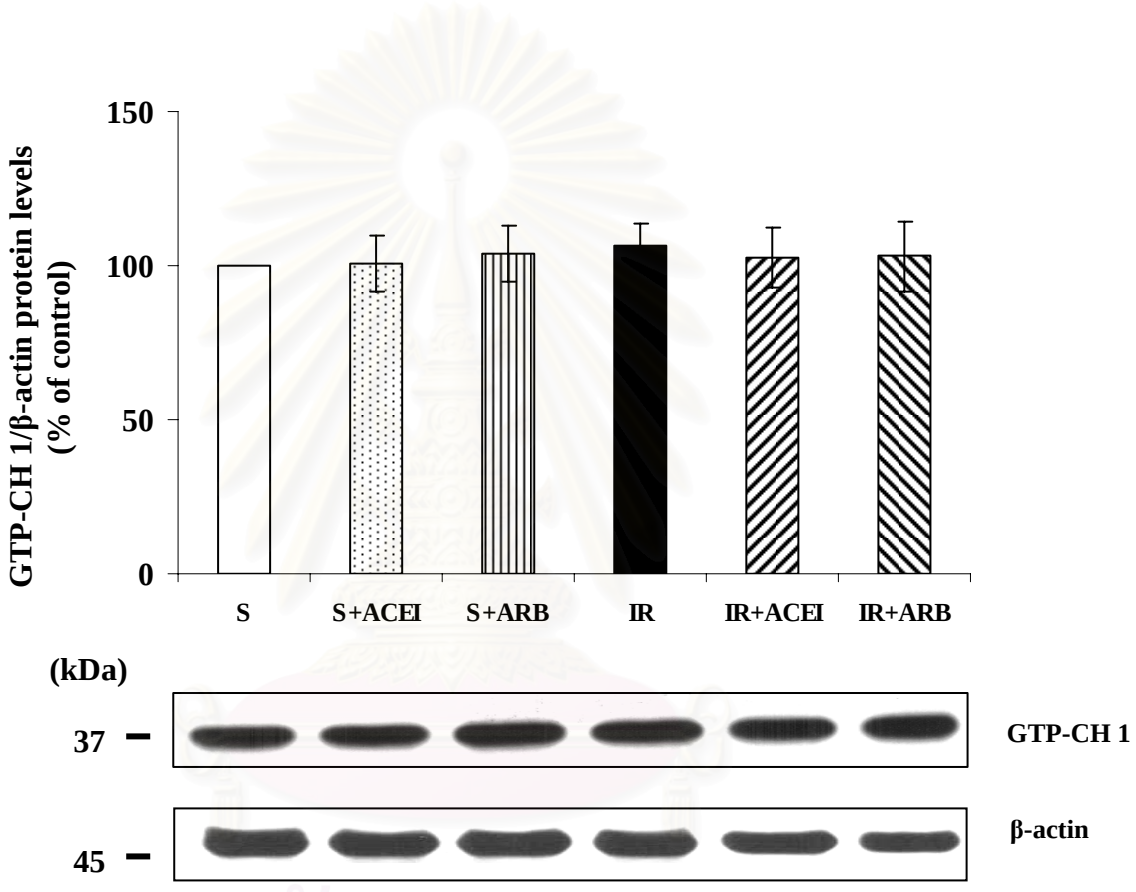


Figure 4: Effects of ACEI or ARB on renal GTP-CH 1 protein abundance in S or IR rats (n = 8/group). The upper panel is histogram bars showing the results of densitometric analyses from pooled data (n = 8/group). Values were normalized by the corresponding optical density for β-actin used as internal control. The lower panel shows the results of immunoblots analyzed in renal tissue, and probed with antibodies against GTP-CH 1 (37 kDa) or β-actin (45 kDa).

Effects of ACEI or ARB on renal eNOS and iNOS mRNA expression in S or IR

For renal eNOS mRNA expression (Figure 5), IR or the inhibition of ANG II did not significantly alter the expression. The mRNA levels were comparable in all groups studied (S = 100 %, S + ACEI = 95.83 $\pm$ 18.13 %, S + ARB = 88.13 $\pm$ 19.99 %; IR = 94.64 $\pm$ 12.38 %, IR + ACEI = 91.46 $\pm$ 13.86 %, IR + ARB = 95.21 $\pm$ 19.68 %).

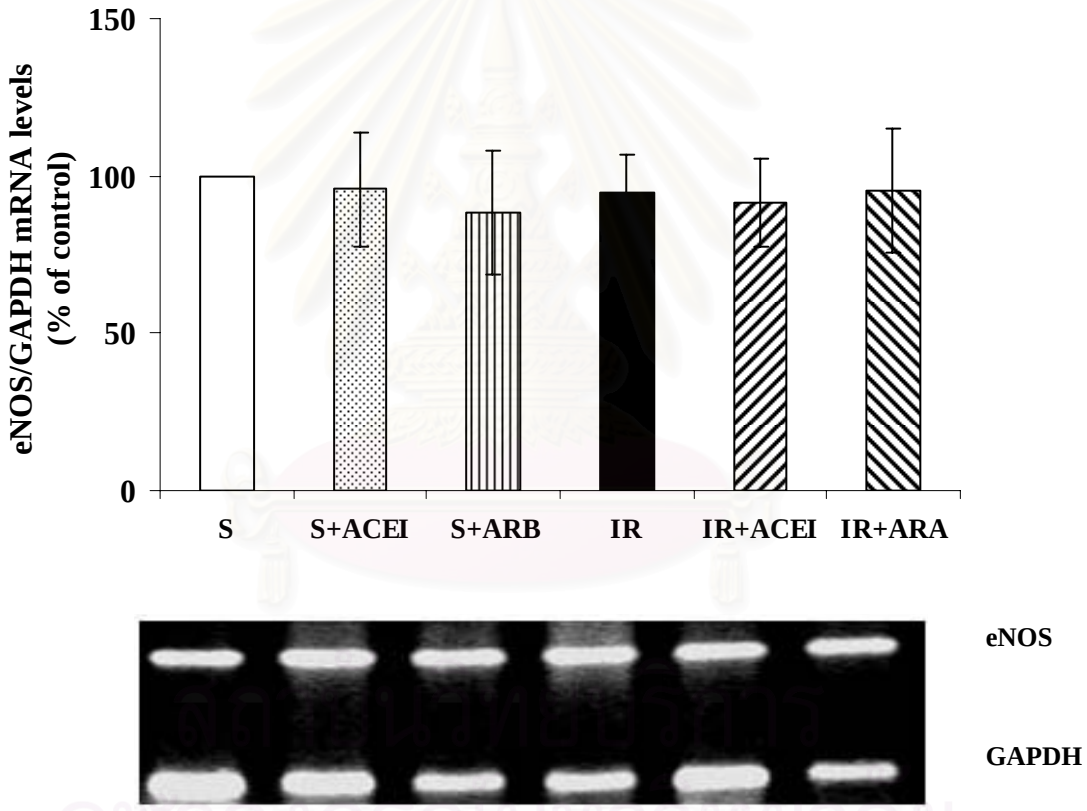


Figure 5: Effects of ACEI or ARB on renal eNOS mRNA expression in S or IR rats (n = 8/group). The upper panel is histogram bars showing the ratio of eNOS to GAPDH intensity. The lower panel is a representative ethidium bromide-stained agarose gel photograph for 515 bp GAPDH mRNA and 210 bp eNOS mRNA.

The renal iNOS mRNA expression (Figure 6) of S rats did not significantly differ from S + ACEI (89.26 ± 20.69 %) or S + ARB (82.33 ± 18.77 %). IR increased the mRNA expression to be 154.35 ± 24.70 % p<0.01) as compared with S or S treated groups. This heightened level was attenuated by ACEI to be 106.25 ± 17.75 % or by ARB to be 110.87 ± 20.46 % (p<0.01).

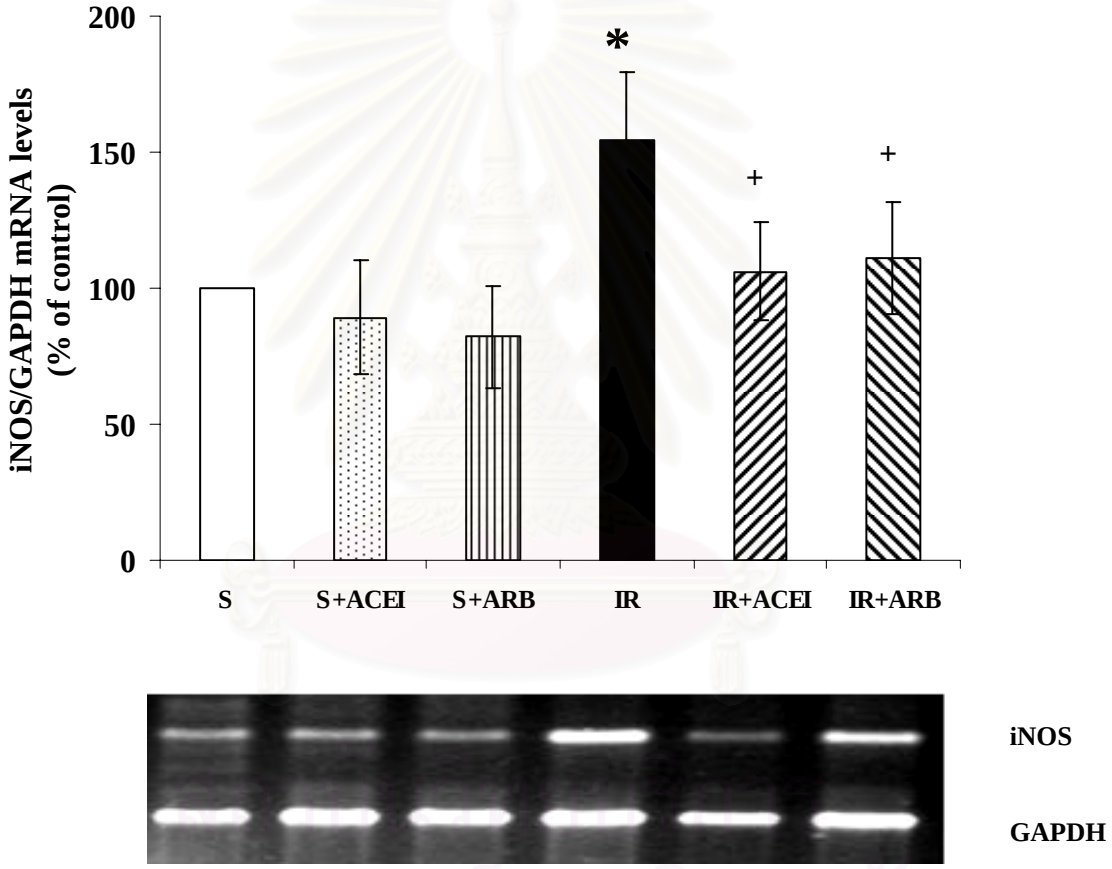


Figure 6: Effects of ACEI or ARB on renal iNOS mRNA expression in S or IR rats (n = 8/group). The upper panel is histogram bars showing the ratio of iNOS to GAPDH intensity. The lower panel is a representative ethidium bromide-stained agarose gel photograph for 515 bp GAPDH mRNA and 272 bp iNOS mRNA. * p<0.01 vs S or S treated groups, + p<0.01 vs IR

Effects of ACEI or ARB on renal eNOS and iNOS protein abundance in S or IR

For total eNOS protein abundance (Figure 7A), the levels did not differ in all experimental groups (S = 100 %, S + ACEI = 100.23 $\pm$ 18.61 %, S +ARB = 94.58 $\pm$ 13.62 %, IR = 91.52 $\pm$ 21.81 %, IR + ACEI = 86.58 $\pm$ 23.58 %, and IR + ARB = 90.80 $\pm$ 13.36 %). However, after immunoblot analysis (LT-PAGE) (Figure 7B), the data showed that eNOS monomer form (135 kDa) was increased from S or S treated groups (S = 100 %, S + ACEI = 101.01 $\pm$ 20.07 %, and S + ARB = 101.08 $\pm$ 19.78 %) to be 205.07 $\pm$ 29.45 % in IR group ($p<0.01$). Either ACEI or ARB could attenuate the heightened levels to be 156.42 $\pm$ 18.72 % by ACEI or to be 154.39 $\pm$ 14.68 % by ARB ($p<0.01$). However, these levels were still significantly higher than S or S treated rats ($p<0.05$).

By contrast, the dimer form was significantly decreased from S or S treated groups (S =100 %, S + ACEI = 106.37 $\pm$ 17.32 %, and S + ARB = 104.72 $\pm$ 15.39 %) to be 65.90 $\pm$ 11.35 % in IR group ($p<0.01$). ACEI or ARB could restore the dimer form of eNOS protein abundance to be 95.76 $\pm$ 19.91 % ($p<0.05$) and 95.70 $\pm$ 16.96 % ($p<0.01$), respectively.

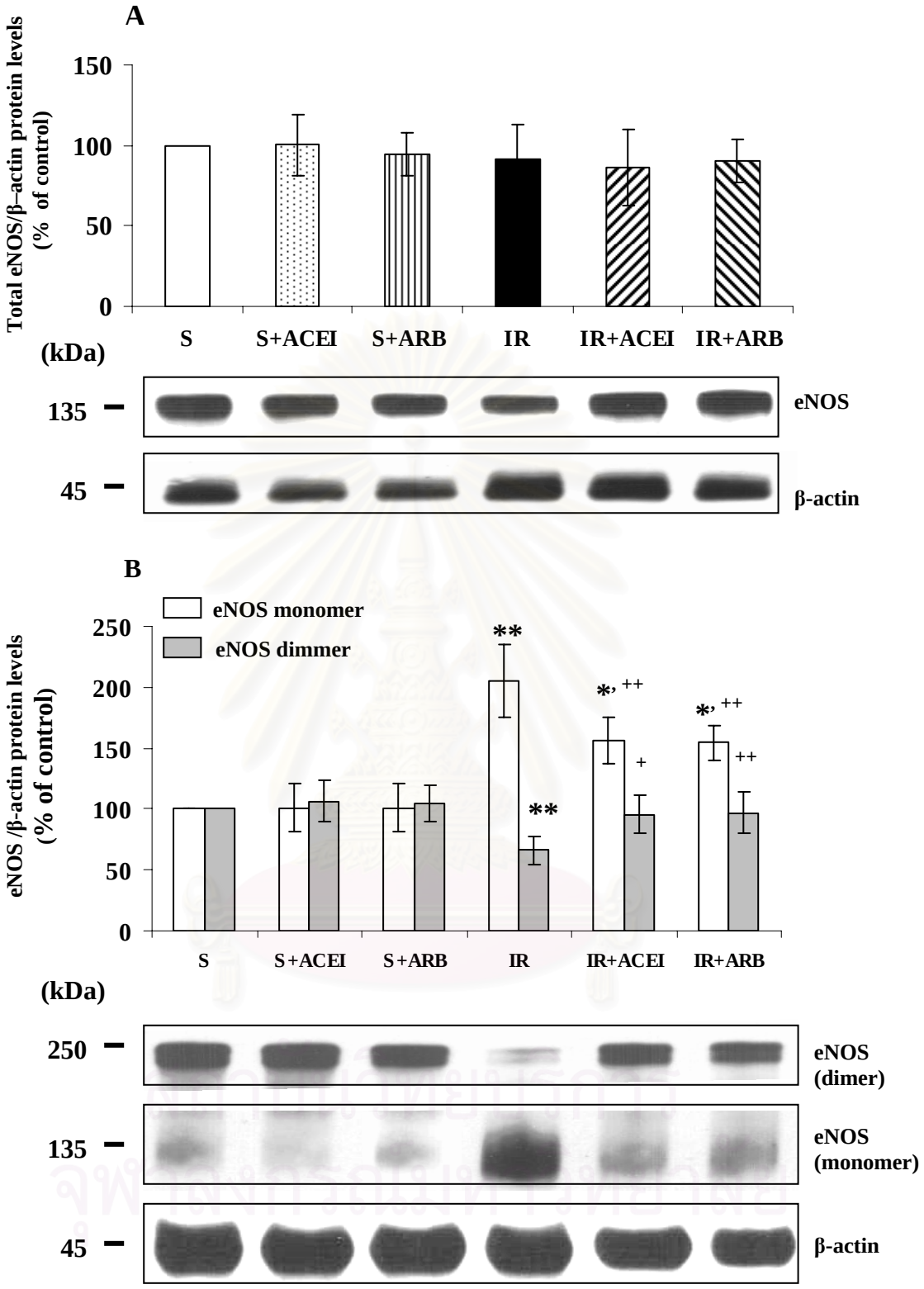


Figure 7: Effects of ACEI or ARB on renal eNOS protein abundant in S or IR rats (n = 8/group). A: Representative the results of immunoblots analyzed for total eNOS protein (135 kDa). B: Immunoblot after LT-PAGE for eNOS monomer and dimer form. * $p<0.05$, ** $p<0.01$ vs S or S treated groups, + $p<0.05$, ++ $p<0.01$ vs respective IR group.

The total iNOS protein expression (Figure 8A) of S rat did not significantly differ from S ± ACEI (99.34 ± 13.91 %) or S ± ARB (96.38 ± 10.23 %). The level significantly increased in IR group to be 193.75 ± 30.10 % ($p<0.01$) as compared with S or S treated groups. Either ACEI or ARB could attenuate the heightened levels to be 128.29 ± 24.91 % by ACEI or to be 132.56 ± 25.10 % by ARB ($p<0.01$).

In immunoblot analysis after low temperature SDS-PAGE (LT-PAGE) (Figure 8B), the immunoreactivity of iNOS monomer form (130 kDa) in S, S + ACEI, and S + ARB were 100 %, 87.84 ± 20.58 %, and 88.85 ± 14.11 %, respectively. The level was evaluated in IR group to be 208.79 ± 8.88 % ($p<0.01$; as compared with S or S treated groups). ACEI or ARB could normalize the heightened level of iNOS monomer form to be 161.82 ± 20.96 % and 160.81 ± 18.16 % ($p<0.01$), respectively. However, these levels were still significantly higher than S or S treated rats ($p<0.01$). Interestingly, the iNOS dimer form did not change significantly in all groups studied (S = 100 %, S + ACEI = 96.57 ± 20.32 %, S +ARB = 87.99 ± 17.92 %, IR = 87.50 ± 16.03 %, IR + ACEI = 105.15 ± 18.69 %, and IR + ARB = 113.24 ± 25.47 %).

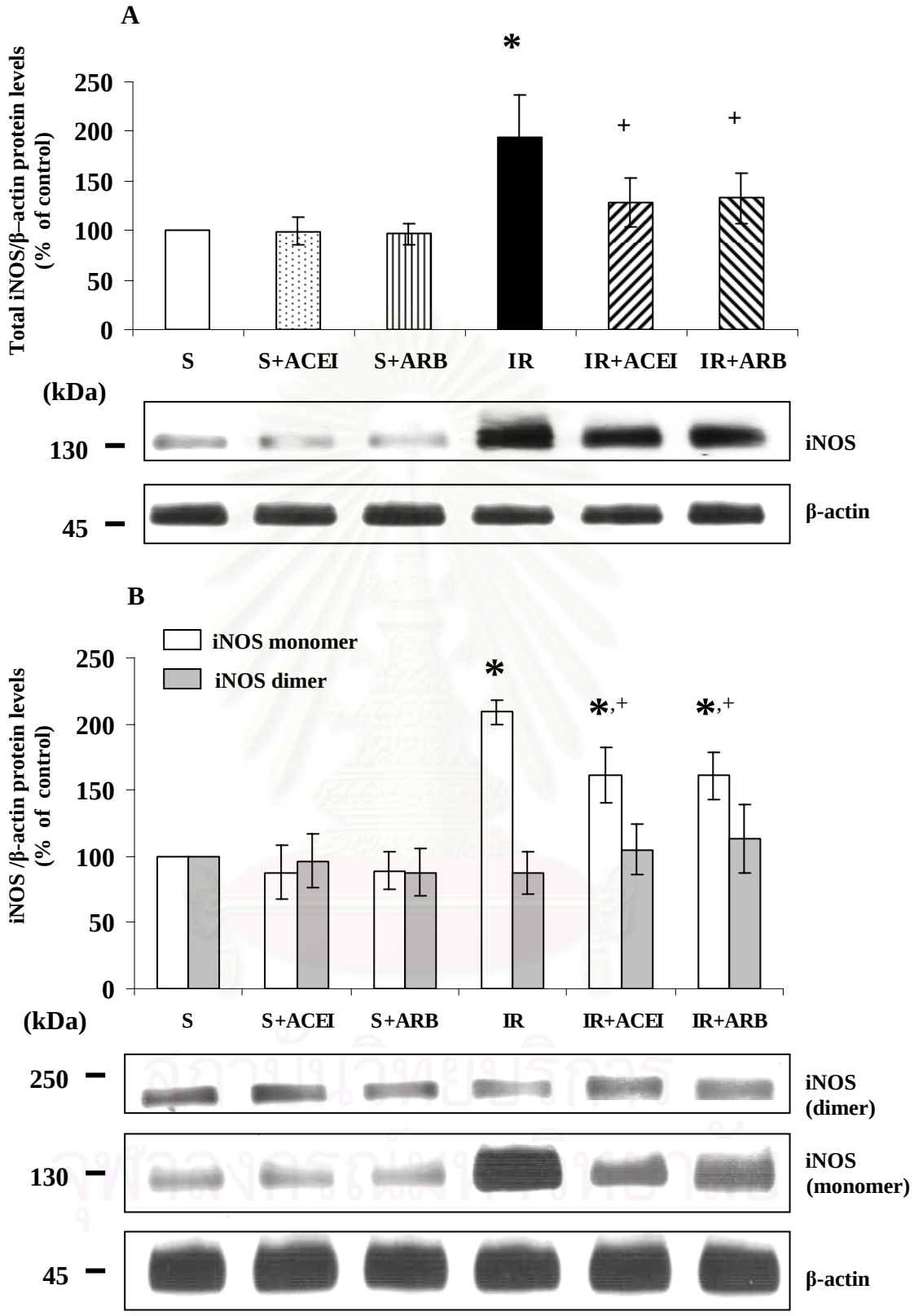


Figure 8: Effects of ACEI or ARB on renal iNOS protein abundant in S or IR rats (n = 8/group). A: representative the results of immunoblots analyzed for total iNOS protein (130 kDa). B: immunoblot after LT-PAGE for iNOS monomer and dimer form. * $p<0.01$ vs S or S treated groups, + $p<0.01$ vs IR

Metabolic Parameters in Serum Chemistry and Renal Function

Serum concentrations of Na^+, K^+, Cl^-, BUN and Cr in all groups studied showed comparable levels (Table 1). As predicted, creatinine clearance and urine flow rate (Table 1) were not significantly altered by IR or treatment with either ACEI or ARB (Table 1).

Table 1: Metabolic and renal function profiles of S and IR rats treated with or without ACEI or ARB[b]

Parameters	S	S + ACEI	S + ARB	IR	IR + ACEI	IR + ARB
Na^+ (mmol/L)	140 ± 1.47	142 ± 1.45	142 ± 1.06	136 ±1.02	138 ±1.02	141 ±1.03
K^+ (mmol/L)	3.39 ± 0.23	4.12 ± 0.21	4.01 ± 0.22	4.06 ± 0.17	4.21 ± 0.16	4.13 ± 0.17
Cl^- (mmol/L)	101 ± 1.63	103 ± 1.61	102 ± 1.16	100 ± 1.17	99 ± 1.16	102 ± 1.18
BUN (mg%)	20.16 ±0.06	24.01 ±0.98	25.11 ±1.01	23.39 ±1.88	25.30 ±1.99	26.71 ±1.12
Cr (mg%)	0.44 ±0.04	0.52 ±0.05	0.50 ±0.04	0.52 ±0.05	0.48 ±0.04	0.56 ±0.05
CCr (ml/min/100gBW)	0.72 ±0.02	0.68 ±0.03	0.76 ±0.06	0.60 ±0.08	0.80 ±0.05	0.62 ±0.07
Urine flow rate $X10^{-2}$ (ml/min)	2.01 ±0.08	1.99 ±0.06	2.04 ±0.05	1.89 ±0.09	2.03 ±0.16	2.19 ±0.09

[b]S, Sham; IR,renal ischemic reperfusion; ACEI, angiotensin converting enzyme inhibitor; ARB, angiotensin II receptor type 1 blocker; BUN, blood urea nitrogen; Cr, creatinine; CCr, creatinine clearance; BW, body weight.

CHAPTER V

DISCUSSION AND CONCLUSION

The results in the present study have demonstrated that IR significantly reduced renal DHFR mRNA and protein expression. Either ACEI or ARB could restore the levels to sham groups. This indicates an essential role of ANG II, which was stimulated in renal IR, in reducing DHFR expression. Of interest, this concurs with a recent study in BAEC, which demonstrated that ANG II could impair DHFR expression (Chalupsky and Cai, 2005). ANG II rapidly and transiently activated NADPH oxidase to produce $O_2^{\bullet}$ leading to H_2O_2-dependent down-regulation of DHFR (Chalupsky and Cai, 2005). In consequence, decreased DHFR levels could induce a deficiency in BH4 which caused NOS uncoupling and production of $O_2^{\bullet}$ instead of NO (Chalupsky and Cai, 2005; Kakoki et al., 2000). Overexpression of DHFR could correct all the above alterations in ANG II-stimulated cells (Chalupsky and Cai, 2005). Moreover, the present study reveales that renal IR with or without ANG II inhibiting agents had no effect on GTP-CH 1 mRNA and protein expression. The results are in agreement with a previous study in non IR model, which showed that endogenous H_2O_2 produced by ANG II did not modulate GTP-CH 1 (Chalupsky and Cai, 2005; Shimizu et al., 2003). Indeed, a recent study illustrated that the exogenous H_2O_2, the magnitude of which was much higher than the endogenous origin, could up-regulate GTP-CH 1 expression and enhanced BH4 synthesis (Shimizu et al., 2003). Taken together, in IR model, DHFR is much more sensitive to endogenous H_2O_2 when compared with GTP-CH 1.

As mentioned earlier, both GTP-CH 1 and DHFR are important enzymes to produce BH4 in the de novo and salvage pathway, respectively (Alp and Channon,

2004; Thony et al., 2000). In this present work, there was no change of renal GTP-CH 1 but renal DHFR level decreased during IR condition. The results imply that BH4 synthesis may reduce, and then could alter NOS function. Therefore, in this study both iNOS snd eNOS expression were measured in the renal tissue simultaneously with DHFR and GTP-CH 1 levels.

For eNOS expression, the present data illustrate that IR or angiotensin inhibition did not alter renal eNOS mRNA and total protein expression. This indicates that IR, in the present study, has no influence on eNOS transcription and translation. However, Kakoki et al. (2000) have demonstrated that total eNOS protein expression significantly increased in rat kidneys subjected to ischemia for 45 min and reperfusion for 1 day. The eNOS mRNA expression was not examined simultanously, though. In deed, the expression of eNOS in ischemia condition are under controversy in which it was enhanced (Valdivielso et al., 2001; Vinas et al., 2006; Zheng et al., 2005) or reduced (Kobayashi et al., 2001; Komurai et al., 2003; McQuillan et al., 1994). This discrepancy seems to be associated with the duration of ischemic period or/and the amount of ANG II being available. For example, an acute ANG II infusion (110 minutes) increased eNOS mRNA in kidney by 70% without changing eNOS protein levels, whereas a chronic infusion (10 days) enhanced eNOS protein by 90% without changing eNOS mRNA levels (Hennington et al., 1998). Moreover, Moreno et al. (2002) have also shown that ANG II infusion for 3 h increased renal cNOS activity without modification of their protein expression while a chronic ANG II (3 d) could augment the protein expression but not the activity. Furthermore, the action of ANG II in the kidney is likely to be area specific regulation on NOS expression. For instance, ANG II administration could up-regulate eNOS mRNA and protein in renal cortex, but not in renal medulla (Chin et al., 1999; Navar et al., 2000).

As with no alteration in the total eNOS protein performed by conventional immunoblot analysis, LT-PAGE revealed that the immunoreactivity for dimeric eNOS was significantly decreased whereas the amount of eNOS monomer was enhanced in IR condition. These present data are similar to those evidences studied by Kakoki et al. (2000). They concluded that BH4 was depleted during IR resulting in eNOS monomerization. The dimer form abundance was restored with BH4 supplementation. Therefore, if the BH4 synthesis does not impair during IR, the amount of BH4 would be sufficient and available for dimerization. This would really occur in the present study since the inhibition of ANG II (by ACEI or ARB) could restore DHFR expression which maintains BH4 levels during IR. Thus, the eNOS dimeric form are stabilized. These present results are in the same manner as those performed by Chalupsky and Cai (2005) that ANG II down-regulated DHFR expression and lowered BH4 levels in endothelial cell culture. The inhibition of ANG II action could converse those alterations.

For iNOS, the present data demonstrate that IR activated renal iNOS mRNA expression which is similar to previous studies (Docherty et al., 2006; Vinas et al., 2006). This may explain by the activating effect of various cytokines induced in IR (Araki et al., 2006; Daha et al., 2000; Donnahoo et al., 1999) or by ANG II (Sahar et al. 2005; Ushio-Fukai et al. 1998; Zoja et al., 1998). According to increase iNOS mRNA, the induction of total iNOS protein ensued. ANG II inhibitors, ACEI or ARB, could attenuate these heightened levels. This indicates that both transcription and translation of iNOS were modified during IR, via ANG II through AT1 receptor. In addition, the inhibition of ANG II would suppress activating cytokines and then reduce iNOS mRNA as well as total protein abundance.

By LT-PAGE, the immunoreactivity demonstrated that IR had no effect on iNOS dimer whereas the monomer was markedly enhanced. This indicates that the monomeric form was accounted for the higher level of total protein. Indeed, the iNOS dimer should be formed more as its monomer forms were ready to be dimerized. This, again, may explain by the depletion of BH4 during IR.

Interestingly, in the presence of ANG II inhibition, the amount of BH4 was not depleted which could promote dimerization. However, the dimeric iNOS proteins were constant. The exact mechanisms of this discrepancy remain unclear. One of the most proposed mechanisms for explanation is the inhibitory and regulatory effect of NO, as a heme iron ligand, a cysteine modifying, and an inhibitor of dimerization. It has been found that NO produced from the catalytic reaction in iNOS not only can rebind to the heme iron, thereby directly inhibiting the turnover of the enzyme (Abu-Soud et al., 1995; Abu-Soud et al., 2001), but also it can induce monomerization of the functional dimers (Chen et al., 2002). Binding of L-arginine to the enzyme shifted the monomer to be the loose dimeric state, whereas BH4 will lock the enzyme in a “tight” dimeric state that resisted to NO-induced monomerization (Li et al., 2006). This NO-induced monomerization in the “loose”-dimer was associated with the formation of an intramolecular disulfide bond between C104 and C109, located in the Zn binding motif in the dimer interface (Li et al., 2006). It was shown that the NO-induced monomers can not be reverted back to the dimeric state by the addition of L-arginine and BH4 (Chen et al., 2002). Another type of dimer inhibition of NO in iNOS has been demonstrated in the RAW 264.7 mouse macrophage cell line (Albakri 1996). In that work, they found that endogenous NO produced by iNOS induced by cytokines down-regulated the formation of iNOS into the dimer by preventing heme insertion and decreasing heme availability despite continued accumulation of the

monomer. The dimer inhibition of NO has been reported in eNOS as well. Ravi et al., (2004) discovered that exogenous NO induced S-nitrosylation of Cys thiol groups in the Zn binding motif causing Zn tetrathiolate cluster and sulfhydryl group destruction at the dimer interface, thereby reducing the dimer level and the associated enzymatic activity. S-nitrosylation has also been reported in iNOS by Mitchell et al. (2005). The autoinhibition by NO is hypothesized as a cytoprotective mechanism to suppress NO overproduction (maintain dimer level) and reduce $O_2^{\bullet}$ generation (inactivate monomer form) (Li et al., 2006). Therefore, during angiotensin inhibition, the slightly higher levels of NOS monomer observed in the present study would be likely not much harmful as predicted since the renal tissue damage and lipid peroxidation induced in IR were lessen when treated with ACEI or ARB (Seujange et al., 2006).

The alternative mechanism that has been investigated intensively is protein inhibitors. It was found that, in macrophage cells, a physiological iNOS specific dimerization inhibitor, designated as NAP110 (NOS-associated protein-110 kDa), was upregulated by IFN-γ and LPS in vivo (Ratovitski et al., 1999a). NAP110 binds to iNOS in the amino-terminus region (residues 1-70), a region that is not homologous to the other isoforms, thereby selectively inhibiting its dimerization. Similarly, Kalirin, a large cytosolic protein with nine spectrin-like repeated, inhibited dimerization of iNOS in neuronal cells by binding in the same region (Ratovitski et al., 1999b). In both cases the protein binds to iNOS monomers but does not convert dimers back into the monomeric form. In view of the potential importance of the dimer inhibitory pathway, a number of highly selective synthetic iNOS dimerization inhibitors have been developed, which show great therapeutic potential in iNOS-related pathologies (Blasko et al., 2002; Kolodziejski et al., 2004). Further studies are needed to elucidate whether these protein inhibitors are also induced in IR condition, or not.

In conclusion, the present study demonstrates the first evidence indicating that IR, via stimulation of ANG II, suppresses renal DHFR but activates iNOS both mRNA and protein expression. IR enhances the monomer while diminishes the dimer form of eNOS. Inhibition of ANG II by using ACEI or ARB could restore DHFR and eNOS dimer whereas attenuate the heightened levels of iNOS expression and NOS monomer. IR has no effect on GTP-CH 1 expression. The present result also indicates that angiotensin II receptor type 1 plays a crucial role in modulation of DHFR and NOS expression.

REFERENCES

Abu-Soud, H. M.; Ichimori, K.; Nakazawa, H. and Stuehr, D. J. (2001). Regulation of inducible nitric oxide synthase by self-generated NO. *Biochemistry* 40: 6876-6881.

Abu-Soud, H. M. and Stuehr, D. J. (1993). Nitric oxide synthases reveal a role for calmodulin in controlling electron transfer. *PNAS*. 90(22): 10769-10772.

Abu-Soud, H. M.; Wang, J.; Rousseau, D. L.; Fukuto, J. M.; Ignarro, L. J. and Stuehr, D. J. (1995). Neuronal nitric oxide synthase self-inactivates by forming a ferrous-nitrosyl complex during aerobic catalysis. *J. Biol. Chem.* 270: 22997-23006.

Abu-Soud, H. M.; Yoho, L. L. and Stuehr, D. J. (1994). Calmodulin controls neuronal nitric-oxide synthase by a dual mechanism. Activation of intra- and interdomain electron transfer. *J. Biol. Chem.* 269(51): 32047-32050.

Albakri, Q. A. and Stuehr, D. J. (1996). Intracellular assembly of inducible NO synthase is limited by nitric oxide-mediated changes in heme insertion and availability. *J. Biol. Chem.* 271: 5414-5421.

Alderton, W. K.; Cooper, C. E. and Knowles, R. G. (2001). Nitric oxide syntheses: structure, function and inhibition. *Biochem. J.* 357(3): 593-615.

Alejandro, V.; Scandling, J. D. Jr. and Sibley, R. K. (1995). Mechanisms of filtration failure during postischemic injury of the human kidney. A study of the reperfused renal allograft. *J. Clin. Invest.* 95: 820-831.

Allred, A. J.; Chappell, M. C.; Ferrario, C. M. and Diz, D. (2000). Differential actions of renal ischemic injury on the intrarenal angiotensin system. *Am. J. Physiol.* 279: F636-F645.

Alp, N. J. and Channon, K. M. (2004). Regulation of endothelial nitric oxide synthase by tetrahydrobiopterin in vascular disease. *Arteioscler. Thromb. and vasc. Biol.* 24: 413-420.

Alp, N. J.; Mussa, S. and Khoo, J. (2003). Tetrahydrobiopterin-dependent preservation of nitric oxide-mediated endothelial function in diabetes by targeted transgenic GTP-cyclohydrolase I overexpression. *J. Clin. Invest.* 112: 725-735.

Andrew, P. J. and Mayer, B. (1999). Review: Enzymatic function of nitric oxide synthases. *Cardiovas. Res.* 43: 521-531.

Aragno, M.; Cutrin, J. C.; Mastrocola, R.; Perrelli, M. G.; Restivo, F. and Poli, G. (2003). Oxidative stress and kidney dysfunction due to ischemia/reperfusion in rat: Attenuation by dehydroepiandrosterone. *Kidney Int.* 64: 836-843.

Araki, M.; Fahmy, N.; Zhou, L.; Kumon, H.; Krishnamurthi, V. and Goldfarb, D. (2006). Expression of IL-8 during reperfusion of renal allografts is dependent on ischemic time. *Transplantation* 81(5): 783-788.

Ardaillou, R. (1999). Angiotensin II receptors. *J. Am. Soc. Nephrol.* 10: S30-S39 (suppl).

Azuma, H.; Ishikawa, M. and Sekizaki, S. (1986). Endothelium-dependent inhibition of platelet aggregation. *Br. J. Pharmacol.* 88: 411-415.

Barrilli, A.; Molinas, S.; Petrini, G.; Menacho, M. and Elias, M. M. (2004). Losartan reverses fibrotic changes in cortical renal tissue induced by ischemia or ischemia-reperfusion without changes in renal function. *Mol. Cell. Biochem.* 260(1-2): 161-170.

Bayraktutan, U. and Ulker, S. (2003). Effects of angiotensin II on nitric oxide generation in proliferating and quiescent rat coronary microvascular endothelial cells. *Hypertens. Res*. 26: 749-757.

Berry, C.; Hamilton, C. A.; Brosnan, M. J.; Magill, F. G.; Berg, G. A. and McMurray, J. J. (2000). Investigation into the sources of superoxide in human blood vessels: angiotensin II increases superoxide production in human internal mammary arteries. *Circulation* 101: 2206-2212.

Bivol, L. M.; Vagnes, O. B. and Iversen, B. M. (2005). The renal vascular response to ANG II injection is reduced in the nonclipped kidney of two-kidney, one-clip hypertension. *Am. J. Physiol.* 289(2): F393-F400.

Blasko, E.; Glaser, C. B.; Devlin, J. J.; Xia, W.; Feldman, R. I. and Polokoff, M. A. et al. (2002). Mechanistic studies with potent and selective inducible nitric-oxide synthase dimerization in hibitor. *J. Biol. Chem.* 277: 295-302.

Bredt, D. S. and Snyder, S. H. (1990). Isolation of nitric oxide synthetase, a calmodulin-requiring enzyme. *PNAS.* 87(2): 682-685.

Brezis, M. and Rosen, S. (1995). Hypoxia of the renal medulla: Its implications for disease. *N. Engl. J. Med*. 332: 647-655.

Cai, H.; Griendling, K. K. and Harrison, D. G. (2003). The vascular NAD(P)H oxidase as therapertic targets in cardiovascular diseases. *Trends. Pharmacol. Sci.* 24(9): 471-478.

Chalupsky, K. and Cai, H. (2005). Endothelial dihydrofolate reductase: Critical for nitric oxide bioavailability and role in angiotensin II uncoupling of endothelial nitric oxide synthase. *PNAS.* 102: 9056-9061.

Channon, K. M. (2004). Tetrahydrobiopterin: Regulator of endothelial nitric oxide synthase in vascular disease. *Trends. Cardiovasc. Med.* 14: 323-327.

Chen, Y.; Panda, K. and Stuehr, D. J. (2002) Control of nitric oxide synthase dimer assembly by a heme-NO-dependent mechanism. *Biochemistry* 41(14): 4618-4625.

Chin, S. Y.; Pandey, K. N.; Shi, S. J.; Kobori, H.; Moreno, C. and Navar, L. G. (1999). Increased activity and expression of Ca^{2+}-dependent NOS in renal cortex of ANG II-infused hypertensive rats. *Am. J. Physiol.* 211: F797-F804.

Cho, H. J.; Martin, E.; Xie, Q. W.; Sassa, S. and Nathan, C. (1995). Inducible nitric oxide synthase: Identification of amino acid residues essential for dimerization and binding of tetrahydrobiopterin. *PNAS.* 92: 11514-11518

Cho, H. J.; Xie, Q. W.; Calaycay, J.; Mumford, R. A.; Swiderek, K. M. and Lee, T. D. (1992). Calmodulin is a subunit of nitric oxide synthase from macrophages. *J. Exp. Med.* 176(2): 599-604.

Chomczynski, P. and Sacchi, N. (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroformextraction. *Anal.Biochem.* 162(1): 156-159.

Crane, B. R.; Arvai, A. S.; Ghosh, D. K.; Wu, C.; Getzoff, E. D.; Stuehr, D. J. and Tainer, J. A. (1998). Structure of nitric oxide synthase oxygenase dimer with pterin and substrate. *Science* 279: 2121-2126.

Curnow, K. M.; Pascoe, L. and White, P. C. (1992). Genetic analysis of the human type-1 angiotensin II receptor. *Mol. Endocrinol.* 6(7): 1113-1118.

Daha, M. R. and van Kooten, C. (2000). Is the proximal tubular cell a proinflammatory cell? *Nephrol. Dial. Transplant.* 15: 41-43 (suppl).

Deng, J.; Kohda, Y.; Chiao, H.; Wang, Y.; Hu, X. and Hewitt, S. M. (2001). Interleukin-10 inhibits ischemic and cisplatin-induced acute renal injury. *Kidney Int*. 60(6): 2118-28.

Docherty, N. G.; Lopez-Novoa, J. M.; Arevalo, M.; Duwel, A.; Rodriguez-Pena, A. and Perez-Barriocarnal, F. (2006). Endoglin regulates renal ischemia-reperfusion injury. *Nephrol. Dial. Transplant.* 21: 2106-2119.

Donnahoo, K. K.; Shames, B. D.; Harken, A. H. and Meldrum, D. R. (1999). Review article: The role of tumor necrosis factor in renal ischemia-reperfusion injury. *J. Urol*. 162: 196-203.

Douglas, J. G. and Hopfer, U. (1997). Novel aspect of angiotensin receptors and signal transduction in the kidney. *Annu. Rev. Physiol.* 56: 649-669.

Fischmann, T. O.; Hruza, A.; Niu, X. D.; Fossetta, J. D.; Lunn, C. A. and Dolphin, E. (1999). Structural characterization of nitric oxide synthase isoforms reveals striking active-site conservation. *Nat. Struct. Biol.* 6: 233-242.

Framer, M. F. and Coen, D. M. (2001). Enzymatic amplification of DNA by PCR: Standard procedures and optimization. In Coen, D. M. (ed), *Current Protocols in Molecular Biology*, pp. 15.1.1-15.1.14. John Wiley & Sons, Inc, USA.

Ghosh, D. K.; Abu-Soud, H. M. and Stuehr, D. J. (1996). Domains of macrophage NO synthase have divergent roles in forming and stabilizing the active dimeric enzyme. *Biochemistry* 35: 1444-1449.

Ghosh, D. K. and Stuehr, D. J. (1995). Macrophage NO synthase: Characterization of isolated oxygenase and reductase domains reveals a head-to-head subunit interaction. *Biochemistry* 34: 801-807.

Gorren, A. C.; Schrammel, A.; Riethmuller, C.; Schmidt, K.; Koesling, D. and Werner, E. R. (2000). Nitric oxide-induced autoinhibition of neuronal nitric oxide synthase in the presence of the autoxidation-resistant pteridine 5-methyltetrahydrobiopterin. *Biochem. J.* 347(Pt 2): 475-484.

Griffith, O. W. and Stuehr, D. J. (1995). Nitric oxide sunthase: properties and catalytic mechanism. *Annu. Rev. Physiol.* 57: 707-736.

Guan, S.; Fox, J.; Mitchell, K. D. and Navar, L. G. (1992). Angiotensin and angiotensin converting enzyme tissue levels in two-kidney, one clip hypertensive rats. *Hypertension* 20: 763-767.

Gunning, M. E.; Ingelfinger, J. R.; King, A. I. and Brenner, B. M. (1996). Vasoactivepeptides and the kidney. In Brenner, B. M. (ed), *The Kidney,* (Fifth edition), pp. 627-712. W.B. Saunders, USA.

Hasegawa, H.; Sawabe, K.; Nakanishi, N. and Wakasugi, O. K. (2005). Delivery of exogenous tetrahydrobiopterin (BH4) to cells of target organs: Role of salvage pathway and uptake of its precursor in effective elevation of tissue BH4. *Mol. Genet. Metab.* 86: S2-S10.

Hattori, Y.; Nakanishi, N.; Kasai, K. and Shimoda, S. I. (1997). GTP-cyclohydrolase I mRNA induction and tetrahydrobiopterin synthesis in human endothelial cells. *Biochin. Biophys. Acta.* 1358: 61-66.

Hemmens, B.; Goessler, W.; Schmidt, K. and Mayer, B. (2000). Role of bound zinc in dimer stabilization but not enzyme activity of neuronal nitric-oxide synthase. *J. Biol. Chem.* 275: 35786-35791.

Hemmens, B. and Mayer, B. (1998). Enzymology of nitric oxide synthases. In Titheradge, M. (ed), *Methods in molecular biology. Nitric oxide protocols* (First edition), pp. 1-32. Humana Press, Totowa, USA.

Hennington, B. S.; Zhang, H.; Miller, M. T.; Granger, J. P. and Reckelhoff, J. F. (1998). Angiotensin II stimulates synthesis of endothelial nitric oxide synthase. *Hypertension* 3: 283-288.

Hevel, J. M.; White, K. A. and Marletta, M. A. (1991). Purification of the inducible murine macrophage nitric oxide synthase. Identification as a flavoprotein. *J. Biol. Chem.* 266(34): 22789-22791.

Ichikawa, I. and Harris, R. C. (1991). Angiotensin actions in the kidney: Reduced insights in to the old hormone. *Kidney Int.* 40: 583-596.

Ito, S.; Arima, S.; Ren, Y. L.; Juncos, L. A. and Carretero, O. A. (1993). Endothelium-derived relaxing factor/nitric oxide modulates angiotensin II action in the isolated microperfused rabbit afferent but not efferent arteriole. *J. Clin. Invest*. 91: 2012-2019.

Jefayri, M. K.; Grace, P. A. and Mathie, R. T. (2000). Attenuation of reperfusion injury by renal ischaemic preconditioning: the role of nitric oxide. *BJU. Int.* 85(9): 1007-1013.

Ji, Z.; Huang, C.; Liang, C.; Chen, B.; Chen, S. and Sun, W. (2005). Protective effects of blocking renin-angiotensin system on the progression of renal injury in glomerulosclerosis. *Cell Mol. Immunol.* 2(2); 150-154.

Kakar, S. S.; Sellers, J. C.; Devor, D. C.; Musgrove, L. C. and Neill, J. D. (1992). Angiotensin II type-1 receptor subtype cDNAs: differential tissue expression and hormonal regulation. *Biochem. Biophys. Res. Commun*. 183: 1090-1096.

Kakoki, M.; Hirata, Y.; Hayakawa, H.; Suzuki, E.; Nagata, D. and Jojo, A. (2000). Effect of tetrahydrobiopterin on endothelial dysfunction in rats with ischemic acute renal failure. *J. Am. Soc. Nephrol*. 11: 301-309.

Kase, H.; Hashikabe, Y.; Uchida, K.; Nakanishi, N. and Hattori, Y. (2005). Supplementation with tetrahydrobiopterin prevents the cardiovascular effects of angiotensin II-induced oxidative and nitrosative stress. *J. Hypertens.* 23: 1375-1382.

Klatt, P.; Pfeiffer, S.; List, B. M.; Lehner, D.; Glatter, O. and Bachinger, H. P. (1996). Characterization of heme-deficient neuronal nitric oxide synthase reveals role for heme in subunit dimerization and binding of amino acid substrate and tetrahydrobiopterin. *J. Biol. Chem.* 271: 7336-7342.

Klatt, P.; Schmidt, K. and Mayer, B. (1992). Brain nitric oxide synthase is a haemoprotein. *Biochem. J.* 288 (Pt 1): 15-17.

Kobayashi, N.; Mori, Y.; Nakano, S.; Tsubakou, y.; Kobayashi, T. and Shirataki, H. (2001). TCV-116 stimulates eNOS and caveolin-1 expression and improves coronary microvascular remodeling in normotensive and angiotensin II-induced hypertensive rats. *Atherosclerosis* 158: 359-368.

Kolodziejksi, P. J.; Koo, J. S. and Eissa, N. T. (2004). Regulation of iNOS by rapid cellular turnover and cotranslational down-regulation by dimerization inhibitors. *PNAS.* 101: 18141-18146.

Komurai, M.; Ishii, Y.; Matsuoka, F.; Toyama, K.; Ominato, M. and Sato, T. et al. 2003. Role of nitric oxide synthase activity in experimental ischemic acute renal failure in rats. *Mol. Cell Biochem.* 244: 129-133.

Kone, B. C. (1997). Nitric oxide in renal health and disease. *Am. J. Kidney Dis.* 30(3): 311-333.

Kontogiannis, J. and Burns, K. (1998). Role of AT1 angiotensin II receptors in renal ischemic injury. *Am. J. Physiol.* 274: F79-F90.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. *Nature* 227: 680-684.

Lamas, S.; Marsden, P. A.; Li, G. K.; Tempst, P. and Michel, T. (1992). Endothelial nitric oxide synthase: molecular cloning and characterization of a distinct constitutive enzyme isoform. *PNAS.* 89(14): 6348-52.

Lane, P. and Gross, S. S. (1999). Cell signaling by nitric oxide. *Semin. Nephrol.* 19: 215-229.

Lerman, L. and Textor, S. C. (2001). Pathophysiology of ischemic nephropathy. *Urol. Clin. North Am.* 28: 793-803.

Levens, N. R.; Gasparo, M.; Wood, J. M. and Bottari, S. P. (1992). Could the pharmacological differences observed between angiotensin II antagonists and inhibitors of angiotensin converting enzyme be clinically beneficial. *Pharmacol. Toxicol.* 71: 241-249.

Li, D.; Hayden, E. Y.; Panda, K.; Stuehr, D. J.; Deng, H. and Rousseau, D. L. (2006). Regulation of the monomer-dimer equilibrium in inducible nitric-oxide synthase by nitric oxide. *J. Biol. Chem.* 281(12):8197-8204.

Lieberthal, W. (1997). Biology of acute renal failure: Therapeutic implications. *Kidney Int.* 52: 1102-1115.

Li, H.; Raman, C. S.; Glaser, C. B.; Blasko, E.; Young, T. A. and Parkinson, J. F. (1999). Crystal structures of zinc-free and -bound heme domain of human inducible nitric-oxide synthase. Implications for dimer stability and comparison with endothelial nitric-oxide synthase. *J. Biol. Chem.* 274(30): 21276-21284.

Li, J. M. and Shah, A. M. (2004). Endothelial cell superoxide generation: Regulation and relevance for cardiovascular pathophysiology. *Am. J. Physiol.* 287: R1014-R1030.

Lowry, O. H.; Rosebrough, N. J.; Farr, A. L. and Randall, R. J. (1951). Protein measurement with the folin phenol reagent. *J. Biol. Chem.* 193: 265-275.

Marsden, P. A.; Hall, A. V. and Brenner, B. M. (2000). Reactive nitrogen and oxygen intermediates and the kidney. In Brenner, B. M. (ed), *The Kidney,* (Sixth Edition), pp. 701-754. W. B. Saunders, USA.

Mattson, D. L. and Wu, F. (2000). Nitric oxide synthase activity and isoforms in rat renal vasculature. *Hypertension* 35(1 Pt 2): 337-341.

Mayer, B. and Hemmens, B. (1997). Biosynthesis and action of nitric oxide in mammalian cells. *Trends. Biochem. Sci.* 22: 477-481.

Mayer, B.; John, M. and Bohme, E. (1990). Purification of a Ca^{2+}/calmodulin-dependent nitric oxide synthase from porcine cerebellum. Cofactor-role of tetrahydrobiopterin. *FEBS. Lett.* 277(1-2): 215-219.

Mayer, B.; John, M. and Heinzel, B. (1991). Brain nitric oxide synthase is a biopterin- and flavin-containing multi-functional oxido-reductase. *FEBS. Lett.* 288: 187-191.

Mayer, B.; Schmidt, K.; Humbert, P. and Bohme, E. (1989). Biosynthesis of endothelium-derived relaxing factor: a cytosolic enzyme in porcine aortic endothelial cells Ca^{2+} dependently converts L-arginine into an activator of soluble guanylyl cyclase. *Biochem. Biophys. Res. Commun.* 164(2): 678-685.

McQuillan, L. P.; Leung, G. K.; Marsden, P. A.; Kostyk, S. K. and Kourembanas, S. (1994). Hypoxia inhibits expression of eNOS via transcriptional and posttranscriptional mechanism. *Am. J. Physiol.* 267: H1921-H1927.

Miller, R. T.; Martasek, P.; Raman, C. S. and Masters, B. S. (1999). Zinc content of Escherichia coli-expressed constitutive isoforms of nitric-oxide synthase. Enzymatic activity and effect of pterin. *J. Biol. Chem.* 274(21): 14537-14540.

Mitchell, D. A.; Erwin, P. A.; Michel, T. and Marletta, M. A. (2005). S-Nitrosation and regulation of inducible nitric oxide synthase. *Biochemistry* 44(12): 4636-4647.

Molitoris, B. A. and Sutton, T. A. (2004). Endothelial injury and dysfunction: Role in the extension phase of acute renal failure. *Kidney Int*. 66: 496-499.

Moreno, C.; Lopez, A.; Linas, M. T.; Rodriguez, F.; Lopez-Farre, A. and Nava, E. (2002). Changes in NOS activity and protein expression during acute and prolonged ANG II administration. *Am. J. Physiol.* 282: R31-R37.

Mundel, P. (1992). Expression of nitric oxide synthase in kidney macula densa cells. *Kidney Int.* 42: 1017-1019.

Murphy, T. J. (1991). Isolation of a cDNA encoding the vascular type-1 angiotensin II receptor. *Nature* 351: 233-236.

Navar, L. G.; Ichihara, A.; Chin, S. Y. and Imig, J. D. (2000). Nitric oxide-angiotensin II interactions in angiotensin II-dependent hypertension. *Acta. Physiol. Scand.* 168: 130-147.

Noble, M. A.; Munro, A. W.; Rivers, S. L.; Robledo, L.; Daff, S. N. and Yellowlees, L. J. (1999). Potentiometric analysis of the flavin cofactors of neuronal nitric oxide synthase. *Biochemistry*. 38:16413-16418.

Olson, S. C.; Dowds, T. A.; Pino, P. A.; Barry, M. T. and Burke-Wolin, T. (1997). ANG II stimulates endothelial nitric synthase expression in bovine pulmonary artery endothelium. *Am. J. Physiol.* 273: L315-L321.

Olson, S. C.; Oeckler, R.; Li, X.; Du, L.; Traganos, F.; Zhao, Z.; and Burke-Wolin, T. (2004). Angiotensin II stimulates nitric oxide production in pulmonary artery endothelium via the type 2 receptor. *Am. J. Physiol.* 287: L559-L568.

Pollock, J. S.; Forstermann, U.; Mitchell, J. A.; Warner, T. D.; Schmidt, H. H. and Nakane, M. (1991). Purification and characterization of particulate endothelium-derived relaxing factor synthase from cultured and native bovine aortic endothelial cells. *PNAS.* 88: 10480-10484.

Raman, C. S.; Li, H.; Martasek, P.; Southan, G.; Masters, B. S. and Poulos, T. L. (1998). Crystal structure of constitutive endothelial nitric oxide synthase: A paradigm for pterin function involving a novel metal center. *Cell (Cambridge, Mass)* 95: 939-950.

Ratovitski, E. A.; Alam, M. R.; Quick, R. A.; McMillan, A.; Bao, C. and Kozlovsky, C. et al. (1999b). Kalirin inhibition of iNOS. *J. Biol. Chem.* 274: 993-999.

Ratovitski, E. A.; Bao, C.; Quick, R. A.; McMillan, A.; Kozlovsky, C. and Lowenstien, C. J. (1999a). An inducible nitric-oxide synthase (NOS)-associated protein inhibits NOS dimerization and activity. *J. Biol. Chem.* 274: 30250-30257.

Ravi, K.; Brennan, L. A.; Levic, S.; Ross, P. A. and Black, S. M. (2004) S-nitrosylation of endothelial nitric oxide synthase is associated with monomerization and decreased enzyme activity. *PNAS.* 101: 2619-2624.

Rhoden, E. L.; Lucas, M. L.; Lima, L. P.; Rhoden, C. R. and Souto, C. A. V. (2001). Effect of L-arginine on the kidney levels of malondialdehyde in rats submitted to renal ischemia-reperfusion. *B. J. U. Int.* 88: 273-277.

Roczniak, A.; Levine, D. Z. and Burns, K. D. (2000). Localization of protein inhibitor of neuronal nitric oxide synthase in rat kidney. *Am. J. Physiol.* 278(5): F702-F707.

Sadjadi, J.; Puttaparthi, K.; Welborn, M. B.; Rogers, T. E.; Moe, O. and Clagett, G. P. et al., (2002). Upregulation of autocrine-paracrine renin-angiotensin systems in chronic renovascular hypertension. *J. Vasc. Surg.* 36(2): 386-392.

Sahar, S.; Dwarakanath, R. S.; Reddy, M. A.; Lanting, L.; Todorov, I. and Natarajan. R. (2005). Angiotensin II enhances interleukin-18 mediated inflammatory gene expression in vascular smooth muscle cells: a novel cross-talk in the pathogenesis of atherosclerosis. *Circ. Res*. 96(10): 1064-1071.

Sawabe, K.; Wakasugi, K. O. and Hasegawa, H. (2004). Tetrahydrobiopterin uptake in supplemental administration: elevation of tissue tetrahydrobiopterin in mice following uptake of the exogenously oxidized product 7,8-dihydrobiopterin and subsequent reduction by an anti-folate-sensitive process, *J. Pharmacol. Sci*. 96: 124-133.

Schwobel, J.; Fisher, T.; Lanz, B. and Mohaupt, M. (2000). Angiotensin II receptor subtypes determine induced NO production in rat glomerular mesangial cells. *Am. J. Physiol.* 279: F1092-F1100.

Seujange, Y.; Kittikowit, W.; Eiam-Ong, S. and Eiams-Ong, S. (2006). Lipid peroxidation and renal injury in renal ischemic reperfusion: Effect of angiotensin inhibition. *J. Med. Assoc. Thai.* 89 (10): 1686-1693.

Sheta, E. A.; McMillan, K. and Masters, B. S. (1994). Evidence for a bidomain structure of constitutive cerebellar nitric oxide synthase. *J. Biol. Chem.* 269: 15147-15153.

Shimizu, S.; Shiota, K.; Yamamoto, S. K.; Miyasaka, Y.; Ishii, M. and Watabe, T. (2003). Hydrogen peroxide stimulates tetrahydrobiopterin synthesis through the induction of GTP-cyclohydrolase I and increases nitric oxide synthase activity in vascular endothelial cells. *Free Radi.l Biol. Med.* 34: 1343-1352.

Siddhanta, U.; Wu, C.; Abu-Soud, H. M.; Zhang, J.; Ghosh, D. K. and Stuehr, D. J. (1996). Heme iron reduction and catalysis by a nitric oxide synthase heterodimer containing one reductase and two oxygenase. *J. Biol. Chem.* 271(13): 7309-7312.

Sorensen, C. M.; Leyssac, P. P.; Salomonsson, M.; Skott, O. and Holstein-Rathlou, N. H. (2004). ANG II-induced downregulation of RBF after a prolonged reduction of renal perfusion pressure is due to pre- and postglomerular constriction. *Am. J. Physiol.* 286: R865-873.

Stuehr, D. J.; Cho, H. J.; Kwon, N. S.; Weise, M. F. and Nathan, C. F. (1991). Purification and characterization of the cytokine-induced macrophage nitric oxide synthase: an FAD- and FMN-containing flavoprotein. *PNAS.* 88: 7773-7777.

Stuehr, D. J. and Ikeda-Saito, M. (1992). Spectral characterization of brain and macrophage nitric oxide synthases. Cytochrome P-450-like hemeproteins that contain a flavin semiquinone radical. *J. Biol. Chem.* 267: 20547-20550.

Tambascia, R. C.; Fonseca, P. M.; Corat, P. D.; Moreno Jr, H.; Saad, M. J. and Franchini, K. G. (2001). Expression and distribution of NOS1 and NOS3 in the myocardium of angiotensin II-infused rats. *Hypertension* 37: 1423-1428.

Tham, D. M.; Martin-McNulty, B.; Wang, Y. X.; Wilson, D. W.; Vergona, R. and Sullivan, M. E. (2002). Angiotensin II is associated with activation of NF-kB-mediated genes and downregulation of PPARs. *Physiol. Genomics* 11: 21-30.

Theuer, J.; Shagdarsuren, E.; Muller, D. N.; Kaergel, E.; Honeck, H.; Park, J. K. et al. (2005). Inducible NOS inhibition, eicosapentaenoic acid supplementation, and angiotensin II-induced renal damage. *Kidney Int.* 67(1): 248-258.

Thony, B.; Auerbach, G. and Blau, N. (2000). Review article: Tetrahydrobiopterin biosyntahesis, regeneration and functions. *Biochem. J*. 347: 1-16.

Ushio-Fukai, M.; Zafari, A. M.; Fukui, N.; Ishizaka, N. and Griendling, K. K. (1996). p22phox is a critical component of the superoxide-generating NADH/NADPH oxides system and regulates angiotensin II-induced hypertrophy in vascular smooth muscle cells. *J. Biol. Chem.* 271: 23317-23321.

Ushio, M.; Alexander, R. W.; Akers, M. and Griendling, K. K. (1998). p38 mitogen-activated protein kinase is a critical component of the redox-sensitive signaling pathways activated by angiotensin II. Role in vascular smooth muscle cell hypertrophy. *J. Biol. Chem.* 273: 15022-15029.

Valdivielso, J. M. (2001). Renal ischemia in the rat stimulates glomerular nitric oxide synthase. *Am. J. Physiol.* 280: R771-R779.

Vargas, A. V.; Robinson, A. V. and Schulak, J. A. (1994). Captopril amelioration of renal reperfusion injury. *J. Surg. Res.* 57: 28-32.

Vinas, J. L.; Sola, A.; Genesca, M.; Alfaro, N.; Pi, F. and Hotter, G. (2006). NO and NOS isoforms in the development of apotosis in renal ischemia/reperfusion. *Free Rad. Bio. Med.* 40: 992-1003.

Walker, L. M.; Walker, P. D.; Imam, S. Z.; Ali, S. F. and Mayeux, P. R. (2000). Evidence for peroxynitrite formation in renal ischemia-reperfusion injury: Studies with the inducible nitric oxide synthase inhibitor L-N^6-(1-iminoethyl) lysine. *J. Phamacol. Exp. Ther*. 295: 417-422.

Waz, W. R.; Van, J. B. and Feld, L. G. (1998). Nitric oxide metabolism following unilateral renal ischemia/reperfusion injury in rats. *Pediatr. Nephrol.* 12: 26-29.

Werner, E. R.; Bahrami, S.; Heller, R. and Werner-Felmayer, G. (2002). Bacterial lipopolysaccharide down-regulates expression of GTP-cyclohydrolase I feedback regulatory protein. *J. Biol. Chem.* 277:10129-10133.

Wong, P. C.; Price, W. A.; Chiu, A. T.; Duncia, J. V.; Carini, D. J. and Wexler, R. R. (1990). Nonpeptide angiotensin II receptor antagonist: VIII. Characterisation of functional antagonism displayed by DuP 753, an orally active antihypertensive agent. *J. Pharmacol. Exp. Therap.* 252: 719-725.

Yamamoto, K.; Nakazawa,N. and Gasegawa, H. (1997). Uptake of tetrahydrobiopterin by cells I monolayer cultures of RBL2H3 and PC12 cell lines. In Pfleiderer, W. and Rokos, H. (ed.), *Chemistry and biology of pteridines and folates,* pp. 759-763. Blackwell Science, Berlin, Germany.

Zheng, J. S.; Wen, Y.; Chen, D. B.; Bird, I. M.; and Magness, R. R. (2005). Angiotensin II elevates nitric oxide synthase 3 expression and nitric oxide production via a mitogen-activated protein kinase cascade in ovine fetoplacental artery endothelial cells. *Biol. Reprod.* 72: 1421-1428.

Zoja, C.; Abbate, M.; Corna, D.; Capitanio, M.; Donadelli, R. and Bruzzi, I. (1998). Phamacology control of angiotensin II ameliorates renal disease while reducing renal TGF-β in experimental mesangioproliferative glomerulonephritis. *Am. J. Kidney Dis.* 31: 453-463.

Zou, A. P.; Wu, F.; and Cowley, A. W. Jr. (1998). Protective effect of angiotensin II-induced increase in nitric oxide in renal medullary circulation. *Hypertension* 31: 271-276.

APPENDIX

RT-PCR AND WESTERN BLOT SPECIFIC BUFFER

Buffers preparations for RT-PCR

10x Ficoll loading buffer 10 ml

Fillcoll	25.00	g
Bromphenol blue	0.025	g
0.5 M EDTA (pH 8.0)	0.20	g

Adjust volume to 10 ml with dH_2O, Store at -20 ºC

1.5% Agarose gel (w/v) 100 ml

Agarose	1.50	g
1x TBE	100.00	ml

Dissolve by heating and occational mixing until no granules are visible.

Add ethdium bromide (stock 10 mg/mi) 10 µl (final concentration 1 µg/ml)

10x TBE buffer (pH 8.0) 1 liter

Tris base	54.00	g
EDTA $2H_2O$ (pH 8.0)	20.00	ml
Slowly add the boric acid, anhydrous	27.50	ml

Adjust the pH to 8.0 with conc. HCl

Adjust the volume to 1 liter with dH_2O

1x TBE buffer 1 liter

10x TBE buffer	100.00	ml
dH_2O	900.00	ml

Adjust the pH to 7.4 with conc. HCl

Adjust the volume to 1 liter with dH_2O

Preparation of the solutions for tris/glycine SDS-polyacrylamide gel electrophoresis for Western blot

Reagents	10% Separating gel (ml)	7.5% Separating gel (ml)	4%Stacking gel (ml)
H_2O	4.67	5.295	3.08
40%acrylamide mix	2.50	1.875	0.50
1.5 M Tris (pH 8.8)	2.50	2.50	1.25
100% Glycerol	0.24	0.24	0.12
20% SDS	0.05	0.05	0.025
10% APS	0.03	0.03	0.01875
TEMED	0.01	0.01	0.00625
Total volume	10.00	10.00	5.00

Buffers preparations for Western blot

1.5 M Tris base (pH8.8) 100 ml

Tris base 18.171 g

dH_2O 80.00 ml

Adjust the pH to 8.8 with conc. HCl and conc. NaOH

Adjust the volume to 100 ml with dH_2O

1 M Tris base (pH6.8) 100 ml

Tris base 12.14 g

dH_2O 80.00 ml

Adjust the pH to 6.8 with conc. HCl and conc. NaOH

Adjust the volume to 100 ml with dH_2O

0.5 M Tris-HCl 100 ml

Tris base	6.00	g
dH_2O	40.00	ml

Adjust the pH to 6.8 with conc. HCl and conc.

Adjust the volume to 100 ml with dH_2O

10% SDS 100 ml

SDS	10.00	g

Adjust the volume to 100 ml with dH_2O

1x Triton lysis buffer 100 ml

25 mM Tris-HCl (pH 8.0)	0.40	g
150 mM NaCl	0.88	g
0.5% Triton X-100	0.50	ml
5 mM EDTA	0.186	g
dH_2O	100.00	ml

10x Laemmli running buffer (pH 8.3) 1 liter

Tris base	30.30	g
Glycine	144.20	g
SDS	10.00	g
dH_2O	900.00	ml

Adjust the pH to 8.3 with conc. HCl and conc.NaOH

Adjust the volume to 1 liter with dH_2O

1x Transfer buffer 1 liter

Tris base	5.80	g
Glycine	2.90	g
SDS	0.37	g
Adjust the volume to 800 ml with dH_2O		
100% Methanol	200.00	ml

10x TBS washing buffer (pH 7.4) 1 liter

Tris base	30.00	g
NaCl	80.00	g
KCl	2.00	g
dH_2O	800.00	ml
Adjust the pH to 7.4 with conc. HCl		
Adjust the volume to 1 liter with dH_2O		

Blocking buffer

5% non fat dry milk in 0.1% TBS (w/v)

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RESEARCH GRANTS Supported by Ratchadapiseksompoth Research Fund, Faculty of Medicine, and the scholarship from The Graduate School, Chulalongkorn University. The Ministry of Education, Thailand.